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Transfer of oxygen isotopes from precipitation to ruminants in managed ecosystems

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Manuscript 1: **^{2}H and ^{18}O depletion of water close to organic surfaces**

Manuscript 2: **Ambient conditions and feeding strategy influence δ^{18}O of milk water in cows (*Bos taurus*)**

Manuscript 3: **Model explanation of the seasonal variation of δ^{18}O in cow *(Bos taurus)* hair under temperate conditions**

Abstract

Background: Oxygen (O) isotopes have been used to trace back the geographic origin in animal tissues or products (e.g. hair or milk) based on the relationship between O isotope composition ($\delta^{18}O$) of animals and that of annual precipitation and the well-known spatial pattern of $\delta^{18}O$ in precipitation. However, the monthly relations were not studied in cows (*Bos taurus*) and three steps of O transfer may distort this relation. (1) The step from precipitation to feed moisture: The flow of liquid water does not involve fractionation and thus soil water (without soil evaporation) and xylem water should be equal to rain water. This may not be the case for adsorbed water if the surface exerts an influence. Such a surface effect would especially be pronounced at low water contents. Also many feed components like hay, concentrates and silage have a low water content and the isotopic composition of their water may also be influenced by such a surface effect. (2) The step from diet (including feed and drinking water) to body water: Various O input fluxes beside dietary water (e.g. air humidity and air O intake) and O output fluxes of animals (e.g. CO_2 and humidity exhalation) distort the isotopic information received from the diet. (3) The step from body water to O in protein like hair keratin involving the body-water-keratin shift. The mechanism of this process is still insufficiently known although some body-water-keratin shifts were established in humans, nonhuman primates and woodrats but none for cows. Additionally, most of studies focus on the long-term (~ annual) isotopic relation between precipitation and animal tissues or products; however the isotopic seasonality in animals, influenced by both feeding strategy and ambient conditions, is indispensible to be considered before a reliable estimation of the origin can be made.

Aims: The aim of this thesis was to investigate the sources of the $\delta^{18}O$ seasonal variation in milk and hair of cows and to detect the parameters influencing the transfer of O isotopes in milk or hair. Specifically, the interests were (1) to investigate the surface effect of organic matters on adsorbed water and to evaluate the influence of this effect on the transfer of isotopic information from precipitation to feed in managed ecosystem; (2) to understand the mechanism of O transfer from feed to body water under different feeding strategies and drinking water sources; (3) to investigate the sources of isotopic variation in hair O and milk water.

Material & Methods: Different organic materials were equilibrated via the gas phase with unconfined water of known isotopic composition to quantify the isotopic difference between adsorbed water and unconfined water. Additionally, the winter soil water at 7 and 20 cm depth in Grünschwaige Experimental Station was sampled and measured, and a model of surface effect was established to calculate the isotopic relationship between total and unconfined soil water.

The O isotope composition of 608 milk samples from 28 farms with various feeding strategies (e.g. grass fed on pasture, stall feeding of cut grass and feeding no fresh grass) and sources of drinking water was measured. A mechanistic model (the newly developed MK model) considering feeding strategy, soil property, ambient conditions and animal physiology was used to describe the $\delta^{18}O$ of milk (δ_{milk}).

The seasonality of $\delta^{18}O$ in tail hair was investigated in a domestic suckler cow that underwent different ambient conditions (temperature and humidity), physiological states (lactating and gravidity) and feeding strategies (pasture feeding and stall feeding) during

five years. Analyzing the hair also allowed including dry periods of the suckler cow. The MK model was also used to describe the seasonal variation of $\delta^{18}O$ in hair (δ_{hair}).

Results & Discussion: The isotopic fractionation between adsorbed water of organic matters and unconfined water became linearly more negative with increasing volumetric solid:water ratio and even exceeded -4 ‰ for O. This surface effect of organic matter did not obviously influence the transfer of isotopic information from precipitation to soil and to plant water in our case because of two reasons: (1) Only mobile water is taken up from the soil by the plants, which is isotopically identical to unconfined (rain) water. (2) In feed stuff the effect became only relevant at extremely low water contents like in hay or concentrates, which contribute only very little to the water intake of ruminants.

The $\delta^{18}O$ of monthly precipitation was significantly ($P < 0.05$) and positively related to that of milk in all strategies. Pasture grass and cut grass strategies exhibited almost the same linear regressions, while the no-grass strategy caused lower δ_{milk}. The mechanistic MK model predicted the seasonal and farm-specific variation of δ_{milk} generally well. However, the predictions by the MK model did not perform better than a simple multiple regression, which was due to the uncertainties originating from farm, precipitation, silage and animal.

The $\delta^{18}O$ of monthly precipitation was also significantly related to that of measured hair. Modelling suggested that three parameters – drinking water, feed internal water and ambient conditions influencing the animal – were the main sources of variation in δ_{hair}. Feed internal water explained more than half of the variation of δ_{hair} within years. Ambient conditions influencing the animal also contributed about half of the variation of δ_{hair} within years. This mechanism may be easily overlooked because of the similarity in

the seasonal variation of feed and body water, both of which are exposed to and transpire in the same ambient conditions.

Conclusions: The transfer of O isotopic information from precipitation to animal body water and animal products involves many processes. It is influenced by feeding strategy, ambient conditions, animal physiology and the source of drinking water, which in industrialized countries may differ from locally derived groundwater. Thus, the relation is not straightforward but the many processes involved could be well represented in a newly developed model, the MK model. Modeling and measurements showed that the body water of a cow reacts quickly to daily changes in ambient conditions and provided feed. On average, however, body water and animal products are closely although indirectly linked to precipitation because precipitation, soil water, water in grass and other feed stuff and the animal itself are influenced by the same parameters, namely air temperature and air humidity including its isotopic composition.

Zusammenfassung

Einleitung: Sauerstoffisotope ermöglichen die Bestimmung der geographischen Herkunft tierischer Produkte, wie Milch oder Haare, anhand des relativen Verhältnisses der Sauerstoffisotopensignatur im Produkt und dem räumlichen Verteilungsmuster der Sauerstoffisotopensignatur des Niederschlags. Zu temporären Veränderungen der Sauerstoffisotopenverhältnisse in Rindern (*Bos taurus*) gab es bisher noch keine Studien. Dabei wird das Isotopenverhältnis des Niederschlags beim Übergang in das tierische Produkt an drei wesentlichen Stellen verändert: (1) Beim Übergang vom Niederschlag ins Trinkwasser und ins Wasser des Futtermittels: Bei der Bewegung flüssigen Wassers kommt es zu keiner Fraktionierung und daher müsste das Bodenwasser (sofern keine Bodenverdunstung stattfindet), das Xylemwasser und das Grundwasser isotopisch gleich dem Regenwasser sein. Dies würde für absorbiertes Wasser nicht mehr gelten, wenn die Oberfläche die isotopische Zusammensetzung beeinflusst. Dieser Effekt müsste besonders bei geringem Wassergehalt ausgeprägt sein. Niedrige Wassergehalte treten auch in vielen Futtermitteln auf (Heu, Silage, Futterkonzentrate), so dass auch deren Wasser durch einen Oberflächeneffekt beeinflusst sein könnte. (2) Beim Übergang vom Trinkwasser und Wasser im Futtermittel in die Körperflüssigkeiten des Tieres (z.B. Milch): Dabei wird das Sauerstoffisotopenverhältnis der Nahrung durch weitere Prozesse der Sauerstoffaufnahme (z.B. mit der Luft und Luftfeuchte) und der Sauerstoffabgabe (z.B. über CO_2 und Luftfeuchte bei der Atmung) verändert. (3) Beim Übergang des Sauerstoffs vom Körperwasser in Proteine, wie beispielsweise Haarkeratin: Die dabei auftretende Fraktionierung ist groß, der Mechanismus bisher jedoch noch nicht ausreichend bekannt. Für Menschen, nichtmenschliche Primaten und Ratten gab es

bereits einige Studien dazu, für Rinder jedoch nicht. Zudem verglichen die meisten Studien langfristige (jährliche) Isotopenverhältnisse zwischen Niederschlag und tierischen Produkten; es ist jedoch unerlässlich jahreszeitliche Änderungen der Isotopensignatur in Tieren, als Folge jahreszeitlicher Änderungen der Fütterungsstrategien und Umgebungsbedingungen, zu berücksichtigen.

Ziele: Ziel dieser Arbeit war es, die Einflussgrößen der jahreszeitlichen Änderungen der Sauerstoffisotopenverhältnisse in tierischen Produkten zu ermitteln und die Parameter, die die Sauerstoffisotope in Milch und Haaren beeinflussen, zu bestimmen. Insbesondere sollte (1) der Oberflächeneffekt von organischem Material auf adsorbiertes Wasser untersucht und sein Einfluss auf den Isotopenfluss von Niederschlag über die Futtermitteln (Gras) in tierische Produkte bewertet werden; (2) der Mechanismus des Sauerstofftransfers vom Futtermittel in das Körperwasser und die dabei auftretenden isotopischen Veränderungen sollten unter Berücksichtigung verschiedener Fütterungsstrategien und Trinkwasserquellen aufgeklärt und quantifiziert werden; (3) dazu sollten die Einflussgrößen der jahreszeitlichen Änderungen des Sauerstoffisotopenverhältnisses von Haaren und von Milchwasser untersucht werden.

Material und Methoden: Um den isotopischen Unterschied von adsorbiertem und ungebundenem Wasser zu ermitteln, wurde organisches Material über die Gasphase mit ungebundenem Wasser bekannter Isotopensignatur bei unterschiedlichen Wasser-Feststoff-Verhältnissen ins Gleichgewicht gebracht. Zudem wurde an der Versuchsstation Grünschwaige im Winter Bodenwasser aus 7 cm und 20 cm Tiefe entnommen und analysiert und ein Modell zur Berechnung der Isotopenverhältnisse von

Gesamtbodenwasser und ungebundenen Bodenwasser unter Berücksichtigung des Oberflächeneinflusses erstellt.

Um den Einfluss von Jahreszeit, Haltungs- und Fütterungsbedingungen auf das Sauerstoffisotopenverhältnis im Milchwasser zu bestimmen wurde das Sauerstoffisotopenverhältnis von den Trinkwasserquellen der Tiere und von insgesamt 608 Milchproben aus 28 landwirtschaftlichen Betrieben mit unterschiedlichen Fütterungsstrategien (Weidefütterung, Stallfütterung mit Schnittgras und ohne Schnittgras) gemessen. Um das Sauerstoffisotopenverhältnis im Milchwasser zu verstehen und zu prognostizieren, wurde ein mechanistisches Modell (das neu entwickelte MK-Modell) verwendet, das die Fütterungsstrategien, Bodeneigenschaften, Umgebungsbedingungen und die Tierphysiologie berücksichtigt. Der Jahresgang des Sauerstoffisotopenverhältnisses in Schwanzhaaren, die etwa ein Jahr an Information sequentiell speichern, wurde über fünf Jahre an einer Mutterkuh untersucht. In dieser Zeit variierten die Umgebungsbedingungen, der physiologischen Zustand und die Fütterungsstrategie der Mutterkuh. Damit konnte auch die Phase des Trockenstehens erfasst werden. Zur Erklärung der jahreszeitlichen Änderungen der Isotopensignatur wurde ebenfalls das MK-Modell verwendet.

Ergebnisse und Diskussion

An organischem Material adsorbiertes Wasser enthielt umso weniger der schweren Sauerstoff- und Wasserstoffisotope gegenüber ungebundenem Wasser je größer das volumetrische Verhältnis von Feststoff zu Wasser wurde. Die Fraktionierung unterschritt für Sauerstoff sogar -4 ‰. Dieser Oberflächeneffekt des organischen Materials veränderte das Bodenwasser gegenüber dem Regen, hatte aber keinen eindeutigen

Einfluss auf den Übergang des Isotopenverhältnisses vom Niederschlag auf das von der Pflanze aufgenommene Wasser, da das stark absorbierte Wasser nicht mobil ist. Der Oberflächeneffekt, nur bei sehr geringem Wassergehalt relevant, beeinflusst das Sauerstoffisotopenverhältnis in relativ trockenen Futtermitteln wie in Heu oder Futterkonzentraten stark. Er ist aber für die gesamte Wasseraufnahme bei Wiederkäuern wenig relevant, da diese Futtermittel nur einen sehr kleinen Anteil dazu beitragen.

Das Sauerstoffisotopenverhältnis des monatlichen Niederschlags und der Milch korrelierte signifikant positiv für alle Fütterungsstrategien ($P < 0.05$). Die linearen Regressionen der Weidegras- und Schnittgrasstrategien stimmten nahezu überein, während für die Strategie ohne Gras geringere Werte gemessen und modelliert wurden. Das mechanistische MK-Modell konnte die jahreszeitlichen und betriebsspezifischen Änderungen der Isotopensignatur von Milch gut wiedergeben. Trotzdem war die Vorhersage des MK-Modells nicht besser als die einer multiplen Regression aufgrund der Unsicherheiten bei den Eingangsgrößen Betrieb, Niederschlag, Silage und Tier.

Das Sauerstoffisotopenverhältnis des monatlichen Niederschlags korrelierte ebenfalls signifikant mit dem der Haare. Die Parameter Trinkwasser, Futtermittelwasser und Umgebungsbedingungen des Tieres hatten im Modell den stärksten Einfluss auf die Isotopensignatur der Haare. Futtermittelwasser und auch Umgebungsbedingungen erklären mehr als die Hälfte der jahreszeitlichen Änderungen des Isotopenverhältnisses der Haare. Der Einfluss der Umgebungsbedingungen des Tieres kann allerdings leicht übersehen werden, da die jahreszeitlichen Änderungen des Isotopenverhältnisses des Wassers im Futtermittel (besonders im Frischgras) und im Körper der Tiere ähnlich sind, da Futtermittel und Tiere denselben Umgebungsbedingungen ausgesetzt sind.

Schlussfolgerungen

Am Übergang des Sauerstoffisotopenverhältnisses vom Niederschlag auf tierische Produkte, wie Milch und Haare sind vielen Prozesse beteiligt. Die stärksten Einflüsse gehen von der Fütterungsstrategie, den Umgebungsbedingungen, der Tierphysiologie aus. Aber auch die Trinkwasserquellen, welche in Industrieländern nicht immer aus lokalem Grundwasser gespeist werden, können modifizierend wirken. Daher ist die Beziehung zwischen dem Sauerstoffisotopenverhältnis im Niederschlag und dem im tierischen Produkt nicht eindeutig. Dennoch konnten die vielen verschachtelten Prozesse gut mit dem neu entwickelten, mechanistischen Modell, dem MK-Modell, wiedergegeben werden. Sowohl die Messungen als auch die Modellierungen zeigten, dass das Wasser im Körper einer Milchkuh schnell auf tägliche Änderungen der Umgebungsbedingungen und der Futtermittel reagiert. Trotzdem sind die Körperflüssigkeiten und die Produkte eines Tieres eng, wenn auch indirekt, mit dem Niederschlag verknüpft, da sowohl der Niederschlag als auch das Bodenwasser, das Wasser in Gras und in anderen Futtermitteln und das Tier an sich von den Parametern Lufttemperatur und Luftfeuchte, einschließlich ihres Isotopenverhältnisses beeinflusst werden.

1. General introduction

1.1 Fundamental knowledge on isotopes

Isotopes, with the same number of protons and different numbers of neutrons, have aroused researchers' interests due to the development of precise measurements on small differences in isotope abundances (McDermott, 2004). Especially, stable isotope analyses of bio-elements have become commonplace in many research disciplines like ecology and environment science (Bowen et al., 2005c; Sulzman, 2007), since they do not undergo radioactive decay and their application is environment-friendly.

The isotope composition of an element in a sample is usually expressed as δ value, which was established by McKinney et al. (1950). The δ value is defined as the deviation of ratios between the rare (heavy) isotope and the abundant (light) isotope relative to the isotope ratio of a standard:

$$\delta = (R_{sample} - R_{standard}) / R_{standard} \qquad \text{(Equation 1.1)}$$

where R is the molar ratio of the heavy to light isotope of the sample and standard. The standards are different for various elements: VSMOW (Vienna Standard Mean Ocean Water) is frequently used as the standard of oxygen (O) and hydrogen (H); the international standards for nitrogen (N), carbon (C) and sulphur (S) are AIR (atmospheric N_2) and V-PDB (fossil marine carbonate, 'Vienna-Peedee Belemnite') and CDT (Canyon Diablo Troilite) respectively. δ is usually reported in units of parts per thousand (per mil, ‰) because the values at natural isotope abundances are rather small.

Isotopes of the same element exhibit different physical and chemical properties, leading to the fractionation after some processes (Sulzman, 2007). These processes involve either equilibrium fractionation reactions (e.g. CO_2 equilibrates with H_2O) or irreversible

kinetic isotope effects (e.g. evaporation, diffusion, dissociation reactions, and enzymatic effects).

The process of isotopic fractionation is quantified with fractionation factors (α), which can be expressed as the ratio of two isotope ratios:

$\alpha_{A-B} = R_A/R_B$ (Equation 1.2)

where R_A and R_B are the isotope ratios of the two substances A and B (usually the substrate and the product). The deviation of the fractionation factor from unity (ε_{A-B} or Δ_{A-B}) is also used to express fractionation:

$\varepsilon_{A-B} = \alpha_{A-B} - 1$ (Equation 1.3)

This value can also be determined from δ value:

$\varepsilon_{A-B} = (\delta_A - \delta_B) / (1 + \delta_B)$ (Equation 1.4)

When δ_B is in the range of natural abundances found for bio-elements in living systems, ε_{A-B} is approximately equal to the isotopic difference between A and B. This is why in food chain studies the correct fractionation value (Eqn 1.4) is often replaced by the simple isotopic difference.

1.2 Isotopic variation in natural and managed ecosystems

Since isotopic fractionation occurs in nature, the spatio-temporal distribution of the isotope composition in atmosphere, hydrosphere and biosphere varies, providing the base for studies in ecology, palaeontology, plant physiology, food authenticity and forensics (Bowen et al., 2005c; Krivachy et al., 2015; Wang and Dickinson, 2012; West et al., 2006). Although the existence of isotopes was already discovered by Nier and Gulbranson in the 1930s (Nier and Gulbranson, 1939), studies involving stable isotope analysis of C, H, O, N and S in managed ecosystems have mushroomed since 1990

(Schnyder and Auerswald, 2008), which opened new avenues to studies on the isotopic elemental flow, nutritional ecology of grassland and cattle production systems.

Of all these fields, the elemental flow in managed ecosystem from ambient sources to animals deserves attention because the understanding of these isotopic elemental flows is a premise of tracing geographic information and dietary history, which are crucial for consumers nowadays due to the frequently global exchange of food (Boner and Forstel, 2004). Thus, the following paragraphs give a brief review of isotopic element flows, transfer processes, associated isotope fractionation and relevant application in managed ecosystems for different bio-elements, with special emphasis on oxygen.

1.2.1 Carbon

For C, the crucial process in nature influencing the isotope composition is the CO_2 fixation during photosynthesis of plants, which prefers ^{12}C over ^{13}C, yielding more depleted $\delta^{13}C$ values in plant organic matters comparing with other pools in the C cycle in ecosystems (Bowen, 2010). The fractionation between CO_2 and C in plant varies considerably, from about 4.4 ‰ in C4 plants to as much as 27 ‰ for C3 plants (Farquhar et al., 1989). The variation of fractionation also exists within C3 and C4 plant types due to the environmental and genetic parameters (Brugnoli and Farquhar, 2000). By knowing the $\delta^{13}C$ of CO_2 and the fractionation caused by CO_2 fixation, the landscape-scale variation, the so-called isoscape of $\delta^{13}C$ in grass can be established. This isoscape created by plants will influence the entire trophic chain within a region including the soil (Auerswald et al., 2009; Wittmer et al., 2010). The plant-derived variation in $\delta^{13}C$ is also reflected in domestic animals with relatively constant fractionation between feed and animal tissues (McCutchan et al., 2003), opening up opportunities to trace the geographic origin of wild and domestic animals based on $\delta^{13}C$ values of their body tissues or

products. Furthermore, the relative contribution of potential diet components can be retrieved by simple mixing models if an animal ingests different feeds with significantly distinguished $\delta^{13}C$ values (Deniro and Epstein, 1978a).

1.2.2 Nitrogen

The isotopic signature of N in soil is influenced by multiple processes occurring in soil, including atmospheric deposition, nitrogen fixation, gaseous losses by ammonia volatilization and denitrification products (NO, N_2O, N_2) and hydrologic leaching of nitrate (NO_3^-). N from biological fixation has a $\delta^{15}N$ close to 0 ‰ (Shearer and Kohl, 1986), while the soil will become more enriched in ^{15}N when depleted products (NO_3, NH_3, N_2O and NO) are removed from the system by leachate and gaseous losses (Hogberg, 1997; Schnyder and Auerswald, 2008). Higher conservation of N under wet and cool conditions may inform as why $\delta^{15}N$ in soil decreases with the increase of humidity and the decrease of annual temperatures (Amundson et al., 2003; Handley et al., 1999) and thus also with altitude on the global and regional scale (Männel et al., 2007). Fertilizer also influences the $\delta^{15}N$ in soil in grassland with organic fertilizers causing higher $\delta^{15}N$ in soil and plants than synthetic fertilizers (Watzka et al., 2006).

Foliar $\delta^{15}N$ is usually depleted comparing with soil organic nitrogen because nitrate and ammonium, the main N sources of plant, exhibit more depleted $\delta^{15}N$ than the source soil organic matter pools, which is caused by the isotopic fractionation during mineralization and nitrification (Pardo et al., 2007).

The nitrogen in animals is almost entirely from diet. Thus, the feed information or its corresponding climatic information is reflected in the protein of animal tissues. For example, $\delta^{15}N$ in hair increases with the decrease of altitude (Männel et al., 2007) and the increase of nitrogen balance surplus (Schwertl et al., 2005). However, ^{15}N in animals is

2 ‰ to 4 ‰ enriched at every trophic level along food chains (Deniro and Epstein, 1981). This trophic level fractionation is due to the excretion of urea and other nitrogenous wastes that are more depleted in ^{15}N than body nitrogen pools (Parker et al., 2005). It has been widely used for deciphering food webs (Auerswald et al., 2010; Cerling et al., 1999; Wada et al., 1991).

1.2.3 Sulphur

S is an important bio-element with many essential functions in living systems although its amount is only below 1 % in plants and below 2 % in animals (Krivachy et al., 2015). The bulk plant S is generally depleted by only 1 – 2 ‰ relative to its primary sources, which are soil, fertilizer sea spray sulfate or SO_2 from the atmosphere (Tanz and Schmidt, 2010). The trophic fractionation of the S isotopes is 1 – 2 ‰ for muscle tissue of herbivores relative to plant diet and the value is 2 – 5 ‰ for keratin materials like hoof and hair (Tanz and Schmidt, 2010). It has been reported that the $\delta^{34}S$ of beef in organic farms was higher than in conventional farms in Europe, which may be caused by the fact that the relatively enriched seaweed was used in organic farms (Schmidt et al., 2005). Furthermore, it has to be noted that a direct identification of geographical origin based on the $\delta^{34}S$ value alone may be quite difficult, because it depends on fraction of local food in the diet. Therefore, most investigations utilize sulfur isotopic signal as one parameter among several elements. However, in contrast to carbon, nitrogen hydrogen and oxygen, which all relate to climatic pattern, sulphur mainly relates to geology and distance to the sea and thus provides a valuable complement to the other bio-elements regarding geographic origin assessment.

1.2.4 H and O in precipitation and plant water

The precipitation is the main source of soil water in grassland ecosystem. It has a well-known pattern in the global scale, primarily due to the isotope effects associated with evaporation and condensation. Globally, the vast majority of atmospheric water vapor is ocean derived (Trenberth et al., 2007) and it is depleted in the heavy isotopes ^{2}H and ^{18}O relative to the liquid water from which it is derived. The general principles showing the difference in the isotope composition of water vapor, δ_E, and the water surface undergoing evaporation, δ_L, were first described by Craig and Gordon (1965):

$$\delta_E = [\alpha_{eq} \times \delta_L - h \times \delta_a - \varepsilon_{eq} - \varepsilon_k] / [(1 - h) + \varepsilon_k / 1000] \qquad \text{(Equation 1.5)}$$

where δ_a stands for the isotope composition of ambient vapor; h is the relative humidity; ε_{eq} is the equilibrium fractionation factor, which is temperature dependent and can also be expressed as $1 - \alpha_{eq}$; ε_k is the kinetic effect, which arises from the transfer of water vapor to a non-saturated atmosphere.

When vapor is formed above the ocean, it subsequently moves land inward and undergoes a progressive rainout process, which is commonly described as a Rayleigh process (Gat, 1996):

$$R_v = R_0 \times f^{(\alpha - 1)} \qquad \text{(Equation 1.6)}$$

where R_v is the isotopic ratio ($^{2}H/^{1}H$ or $^{18}O/^{16}O$) of residual vapor at a certain point in time, R_0 is the initial isotope ratio of the vapor reservoir, f is the fraction of vapor remaining, and α is the temperature-dependent equilibrium fractionation between condensed rain and vapor, which does not contain the kinetic fractionation because the condensation happens under saturated condition.

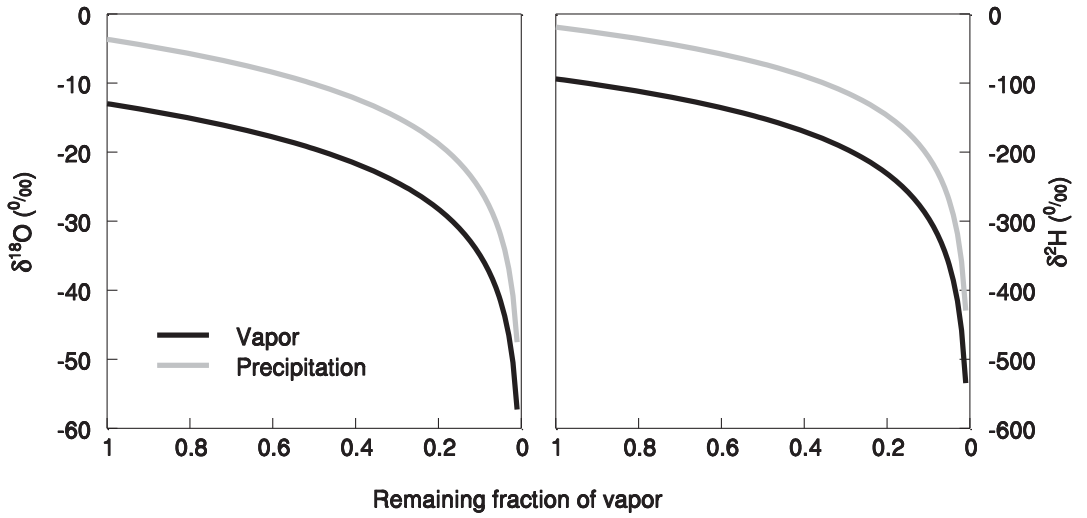

Figure 1: Changes in $\delta^{18}O$ and $\delta^{2}H$ of precipitation and vapor at 25 °C according to a Rayleigh distillation. The initial isotope composition of vapor is set to be -13 ‰ and -94 ‰ for O and H, respectively.

Heavier isotopes accumulate in the liquid phase and will preferentially be removed by precipitation. This procedure decreases their fraction in remaining vapor and the vapor will become isotopically more depleted over time, producing more depleted precipitation with the progressive process of "rainout" (Equation. 1.6 and Fig. 1).

The rainout process causes a depletion of heavy isotopes in precipitation with the increasing distance from the coast but on the global scale there are also influences of latitude and altitude (Bowen, 2010). The latitude effect is about -0.6 ‰ per degree of latitude for O in continental countries in Europe and North America, and can even reach −2 ‰ in the colder Antarctic continent (Bowen and Revenaugh, 2003). The altitude effect ranges between 0.1 and 0.5 ‰ per 100 meters for O and primarily results from the cooling of the air masses as they rise to a mountain (Sulzman, 2007). The inland effect, combining the effect of latitude, rainout and altitude, varies considerably from area to

area and from one season to another. In the western and central Europe the inland effect is about -3.8 ‰ per 1000 km for O while in the further east the effect is only -1.6 ‰ per 1000 km for O (Rozanski et al., 1993). The inland effect in summer is only about one fourth of that in winter at the continent from the Irish coast to the Ural Mountains (Rozanski et al., 1993). Rain amount is another effect that influences the isotope composition of precipitation. This effect is related to the equilibration with air close to the earth surface when the rain drop falls: small drops fall slowly and equilibrate quickly with the warm air close to the earth surface, creating a relatively enriched rain event (Lee and Fung, 2008) while large drops fall quickly and preserve their depletion resulting from cool temperatures found at large altitude. Furthermore, there is also seasonal variation. Generally, the precipitation is more enriched in ^{18}O in summer than in winter, which is mainly caused by the influence of both temperatures in the source region of the water vapor and the place where the precipitation happens (Buenning et al., 2012). This seasonality is different in different places: the continental areas exhibit much higher seasonal variations than in marine or coastal regions. This is caused by the difference in the degree of rain-out, evapotranspiration over the continents and seasonal changing of vapor sources (Rozanski et al., 1993).

The latitudinal, altitudinal, continental, amount and seasonal effect create the spatial and temporal variation in the isotopic signal of precipitation on the global scale. This information, more or less, transfers to soil water and then to plant root without any isotopic fractionation, with exception of some xerophytic and halophytic species (Ellsworth and Williams, 2007). The isotopic signal of water in grass leaves reflects partly that of soil water but is also modified by the fractionating transpiration process

(Farquhar and Cernusak, 2005). The transpiration generally also follows the Craig-Gordon equation (equation 1.5) with a little difference in the calculation of ε_k:

For O: $\varepsilon_k = (32 \times r_s + 21 \times r_b) / (r_s + r_b) / 1000$ (Equation 1.7)

For H: $\varepsilon_k = (25 \times r_s + 17 \times r_b) / (r_s + r_b) / 1000$ (Equation 1.8)

where the values 32 and 21 in Equation 1.7 are O fractionation factors due to the diffusion of water molecules through the stomata and boundary layer, respectively. The values 25 and 17 in Equation 1.8 are those same fractionation factors for H. r_s and r_b represent the stomatal and boundary layer resistances to diffusion of water vapor, respectively. They are the inverses of the stomatal and boundary layer conductances.

Equations 1.5 and 1.7 or 1.8 estimate the general trends in leaf water enrichment quite well, however, in some cases the measured values are different from the estimated ones. In order to improve the estimation, some studies also accounted for the influences of more parameters like the convection of unenriched water towards the sites of evaporation opposed by back diffusion of enrichment from these sites (Barbour, 2007), the diurnal changes in the evaporative environment (Cernusak et al., 2002) and the unenriched water within veins (Roden and Ehleringer, 2000).

1.3 Isotopes in animal tissues and products

1.3.1 Distribution among products and tissues

The general principle for the isotopic signal in animals is: you are what your eat (DeNiro and Epstein, 1978b). This is not fully true however, if excretion causes fractionation. This is why DeNiro et al. (1978) added: plus a few permil. This is the base for trophic level assessment by measuring ^{15}N (Post, 2002). This “few permil” does not apply anymore for water because of the influences of fractionations of different output fluxes. For example,

^{18}O in CO_2 production is usually 38 ‰ higher than in body water, while the transcutaneous vapor is more depleted in ^{18}O comparing with the body water (Kohn, 1996).

Moreover, the general rule only applies for the entire animal (e.g., the whole cow) but not for parts of it. Some substances or tissues deviate from each other due to chemical fractionation or a preferential synthesis from a certain feed component. For example, ^{13}C in whole milk of cows is reported to be 0.4 ‰ depleted than diet due to a strong (2.2 ‰) ^{13}C-depletion of fat, while casein and lactose are 1.1 ‰ and 0.7 ‰ more enriched than diet. Faeces are 1.7 ‰ depleted in ^{13}C (Schneider et al., 2015). In contrast, carbonate in bones and teeths is generally enriched in ^{13}C between 9 ‰ and 15‰ relative to diet (Sulzman, 2007). Also for ^{15}N, this enrichment varies among tissues due to the difference of the available source compounds in metabolic pool and of tissue-specific N fractionation (Gannes et al., 1998; Gannes et al., 1997). For S, it has been reported that the muscle in cows was 0.6 ‰ more enriched than diet, while the bone and cartilage collagens were 0.5 and 1.7 ‰ depleted than diet, respectively (Nehlich, 2015). For O, it is also true that the values are significant different in liquid and solid. For instance, O in phosphate is 17 to 28 ‰ more enriched than the body water (Kohn, 1996) and O in hair is about 15 to 17 ‰ more enriched than body water (O'Brien and Wooller, 2007; Podlesak et al., 2008).

Furthermore, this general rule only applies for the entire animal under equilibrium conditions. The turnover of isotopes, actually, varies in different tissues (Braun et al., 2013; Sulzman, 2007). For N, the half-life in feces was short (10 h), while the value can reach 20 h in casein in milk; For C, the value was 9 h in milk, while it was relatively long in hair (14 h) (Braun et al., 2013; Schwertl et al., 2003). For the determination of

fractionation constants and many other questions it is thus advantageous if the animal is in isotopic equilibrium with its feed by feeding the same feed for a sufficient length of time which may even cover the preceding generation. For O, although it is generally thought that body water is only one well-mixed pool (Kohn, 1996), such near-constant conditions that facilitate analyses cannot be achieved due to the complex variation of amount and isotope composition in input and output fluxes caused by the variations of ambient conditions, of animal physiology and of feeding.

1.3.2 H and O fluxes into and out of animals

The δ^2H of body water is influenced by the input fluxes (chemically bound H in feed, feed moisture, drinking water, inhaled air water vapor) and output fluxes (fecal water, milk water, exhaled water vapor, sweat water, urine water, transcutaneous water vapor, organic products and urea). The resulting δ^2H in body water, subsequently but only partly, exchanges with organic matter during biosynthesis, providing opportunities for the H isotopes of body water to become incorporated into proteins, carbohydrates, and fatty acids in tissues. Most studies on δ^2H focused on the isotopic relationship between δ^2H in keratin of hair or hoof and precipitation. A model was built to describe the sources of H in hair: the H isotopes in keratin are partly from H that fully exchanges with water in the hair follicle during the synthesis of keratin and, also, from C-bound H, which is from both essential and unessential amino acids (Ehleringer et al., 2008). Essential amino acids still reflect the δ^2H values of the food sources while unessential amino acids, if synthesized within the animal, represent body water. Despite this combination of essential and non-essential amino acids, the δ^2H in keratin is strongly correlated with δ^2H in precipitation in many species (Chamberlain et al., 1997; Cryan et al., 2004; Ehleringer et al., 2008).

For O, more fluxes are involved. The input fluxes comprise chemically bound O in feed, feed moisture, drinking water, inhaled air water vapor and air O and output fluxes comprise CO_2, fecal water, milk water, sweat water, urine water, exhaled water vapor, transcutaneous water vapor, organic products and urea. In turn these fluxes are influenced by many parameters.

Chemically bound O: In ruminant feed it originates mainly from carbohydrates, which are primarily made of cellulose. Current knowledge on the enrichment of ^{18}O in cellulose relative to source water ($\Delta^{18}O_{Cel}$) has been summarized in a quantitative model by Barbour (2007):

$$\Delta^{18}O_{Cel} = (1 - p_{ex}p_x) \times \Delta^{18}O_{LW} + \varepsilon_o \quad \text{(Equation 1.9)}$$

where $\Delta^{18}O_{LW}$ is the ^{18}O enrichment of bulk leaf water relative to source water; ε_o (about 27 ‰) is the equilibrium fractionation factor between carbonyl oxygen and water; p_x is the proportion of source water at the site of cellulose synthesis and p_{ex} is the proportion of exchangeable oxygen in cellulose formed from simple carbohydrates. A general value of 0.4 is usually used as a default $p_{ex}p_x$, if no further information is given (Cernusak et al., 2005; Liu et al., 2016). However, in reality, the value of $p_{ex}p_x$ potentially varies from 0.1 to 0.9 (Song et al., 2014), which is influenced by futile cycling of hexose through triose phosphates or turnover of non-structural carbohydrate pools (Liu et al., 2016).

Feed moisture: It originates mainly from grass and/or silage for domestic ruminants. Water in grass is stem and leaf water but on some days dew or rain may also adhere to grass. It has already been found the $\delta^{18}O$ of silage water relates to the conditions during silage production but also the atmospheric conditions during exposure in the feed bunk (relative humidity, exposure time, and the $\delta^{18}O$ of the air vapor) influence the $\delta^{18}O$ of silage water (Sun et al., 2014). Besides the influence of $\delta^{18}O$ in individual feed, the

proportions of each type of feed moisture (grass, silage and intercepted water) exert a large influence on the $\delta^{18}O$ of feed moisture.

Drinking water: In most cases, drinking water is taken from groundwater, which, more or less, reflects the mean annual precipitation. There is usually no significant seasonal fluctuation of $\delta^{18}O$ in groundwater due to its mean age usually covering decades to centuries. In USA and China, the mean standard deviation of groundwater within one year is only 0.2 and 0.9 ‰, respectively (Kennedy et al., 2011; Zhao et al., 2017). However, the different depths of the groundwater stories may carry different isotopic signals due to the different geographic origin and the different age of the water. The $\delta^{18}O$ of pre-Holocene groundwaters in deep stories, especially those recharged during the end of the Pleistocene deglaciation, is significantly lower than those of younger precipitation and the resulting groundwater (Bowen et al., 2007). Dassi et al. (2005) found that the deep groundwater in Tunisia, recharged probably during the late Pleistocene and the early Holocene periods, is about 1.5 ‰ more depleted than shallow ground water. Furthermore, drinking water is, sometimes, taken from rivers or lakes, which then is more enriched than precipitation because of evaporation.

Air vapor: It is usually thought to be equilibrated with precipitation. It also exhibits the same seasonal and spatial variations and a global pattern like the precipitation.

Air O: It has a nearly constant value of 23.5 ‰ across the world (Kohn, 1996). However, lungs prefer up taking ^{16}O. The $\delta^{18}O$ of air O utilized in the lungs is thus influenced by the O utilization fraction (15 to 25 % for terrestrial animals) and the Z-factor (9 to 12 ‰) which is affected by blood hemoglobin content.

The O in CO_2: Its $\delta^{18}O$ depends on the $\delta^{18}O$ in body water and a fractionation that is temperature dependent. The fractionation is 38 ‰ at typical mammal body temperatures (Podlesak et al., 2008).

Fecal water, milk water, sweat water and urine water: They are all formed from body water without obvious fractionation. Abeni et al. (2015) found the O isotopic fractionation between plasma and fecal water to vary between -0.5 ‰ and +0.5 ‰, fractionation of urinary water to vary between -3.3 ‰ and + 1.8 ‰, and the fractionation of milk water to vary between -0.3 ‰ and -0.4 ‰ in pluriparous cows. Wong et al. (1988) found no significant fractionation between plasma and urinary water or saliva in humans. Schoeller et al. (1988) also found that the sweat water and urinary water were both unfractionated in humans. However, the amounts of fecal water, sweat water and urine water vary largely with ambient conditions. This is especially obvious for sweat water: a higher amount of sweat water is excreted in summer than in winter. The amount of milk water varies with the stage of lactation and the production intensity (breed, age, feeding).

Exhaled water vapor: Exhalation through the mouth releases vapor that is equilibrated with body water at body temperature, producing a fractionation of -8 ‰ relative to body water, while the nasally exhaled vapor is subject to fractionation at a temperature that is approximately half-way between body temperature and air temperature (Langman et al., 1979), yielding an average fractionation of -17 ‰ for ruminants (Kohn, 1996). Furthermore, the amount of exhaled water varies: the amount rises under hot conditions by panting, which is used for cooling.

Transcutaneous vapor: It fractionates from -8 ‰ to -21 ‰ relative to body water (Podlesak et al., 2008; Schoeller et al., 1986), depending on ambient conditions and skin temperature. The commonly used value is -18 ‰ (Kohn, 1996). In addition, the amount

of transcutaneous vapor varies according to the ambient conditions: under heat stress, the value increases significantly (Maia et al., 2005).

Organic products: Some O leaves the body water to form organic products. They are primarily involved in milk production and growth of weight. The O fractionation between dry milk and milk water ranges between 14 ‰ and 16 ‰ (Bontempo et al., 2012). The fractionation between body water and organic products contributing to body weight is unknown. However, it should be influenced by the chemical composition of the products, the phosphate O in bone is about 17.5 ‰ more enriched than body water while the O in carbonate is 27 ‰ more enriched (Kohn, 1996; Krivachy et al., 2015). The O in proteins in milk is 15 ‰ more enriched than body water on average (Bontempo et al., 2012).

1.3.3 Keratin archives

Among different tissues and products, hair is frequently researched in forensic investigations, origin tracing and diet identification because it grows continuously and preserves its isotopic information once formed (Schwertl et al., 2003). Except for small amount of lipids and other matter discharged from secretory glands, hair is mainly made up of keratin, which is a S-rich protein structure formed from follicle and the nutrition is supplied by blood capillaries (Popescu and Hocker, 2007). The information of body water or diet can be, at least partly, reflected in the keratin.

1.4 Geographic assessment - The distortion of isotopic signal from precipitation to hair O

The isotopic information of precipitation will be distorted when it enters the hair of mammals. This distortion is influenced by many parameters that can be grouped into ambient conditions, feeding and keeping conditions, animal physiology and sources of

drinking water. This distortion will make the assessment of geographic information difficult. In this thesis I will investigate the O isotope transfer from precipitation to hair and mainly focus on the isotopic distortions that may happen during the following steps of transfer: (1) from precipitation to feed water; (2) from diet (including feed and drinking water) to body water; (3) from body water to hair O.

During the first step, the isotopic information of precipitation does not always enter into the feed water for cows because the feed water can hardly reflect all the precipitation information not only because of the transpiration of feed water but also possibly due to the fractionation effect influenced by the organic surface of soil or feed. There may be two layers at the surface: a thin layer (inner layer) that is in direct contact and influenced by the surface of the solid and a second layer (outer layer) of varying thickness depending on the total moisture content. If the $\delta^{18}O$ of water in these two layers differ and the outer layer was influenced by the air humidity, the isotopic signal of soil water and feed water may not indicate that of precipitation properly. There are some indirect evidences from studies of plant water uptake from soil, which suggests that mobile water in soil differs isotopically from immobile water (Brooks et al., 2010; Evaristo et al., 2015; Tang and Feng, 2001) but this effect has only been directly studied for clay (Oerter et al., 2014) and silica surface (Richard et al., 2007). It is still unknown how large is this surface effect for organic matter and weather this effect in organic matter influences the transfer of isotopic signal from precipitation to feed water or soil water.

In the second step, from diet to body water, the isotopic composition of body water is influenced by a large number of parameters, which may further distort the precipitation signal. Firstly, a significant seasonal variation of $\delta^{18}O$ in body water has been found (Abeni et al., 2015; Boner and Forstel, 2004; Camin et al., 2008; Kornexl et al., 1997).

This suggests the regional variation of the body water isotopes may be masked by the seasonal variation. This makes the identification of origin arduous if the production date of body water (e.g. milk) is unknown. Secondly, the $\delta^{18}O$ of body water is influenced by both O input and output fluxes which are directly and indirectly affected by ambient conditions, animal physiology and even feeding strategy (Boner and Forstel, 2004; Kohn, 1996). The contribution of each flux to $\delta^{18}O$ of body water must be investigated before a precise tracing of the origin. Although there are some models describing the $\delta^{18}O$ of body water in different species based on O balance (Bryant and Froelich, 1995; Gretebeck et al., 1997; Kohn, 1996; Podlesak et al., 2008), these models do not consider the influence of ambient conditions (such as temperature and humidity) and feeding strategy or did not focus on the seasonal variation of $\delta^{18}O$ in body water. A new model linking ambient conditions, animal physiology and feeding strategies is necessary to interpret the complicated sources of isotopic variation in body water.

For the third step, from body water to hair, a model applied in humans, nonhuman primates, and woodrats has been developed (Bowen et al., 2009; Ehleringer et al., 2008; O'Grady et al., 2012). The model assumes that the $\delta^{18}O$ in hair is derived from isotopic exchange with gut water during hydrolysis of dietary protein to form amino acids and the gut water, in turn, results from the mixture of food water, drinking water and body water. After adsorption through the gut wall, amino acids O experience little exchange. During following protein synthesis to produce keratin, the O atoms in amino acids either turn into carbonyl O in the protein or become part of the by-product water. Hence, the protein in hair reflects the information in gut water. Nevertheless, the specific mechanism for domestic animals is still unknown. Different from other monogastric animals, cows have a four-compartment stomach with different functions involved in both water absorption

and remixing. Saliva mixing with feed and drinking water first enters in to reticulorumen and exchanges with body water by the wall of reticulorumen. Then the digesta enters in to the omasum, and also part of drinking water enters the omasum by bypass flow through the esophageal groove without mixing with the water in rumen. Subsequently, the digesta from omasum moves to abomasum and mixes with gastric acid. The absorption and remixing make the prediction of gut water increasingly complicated than in monogastric animals.

The basic aims of this thesis are to analyze the isotopic signal transfer from precipitation to animals in managed ecosystem and to understand the multiple influences of ambient conditions, feeding strategy and animal physiology on the O isotopic signal in ruminant animals taking into account the specific conditions during these three steps.

In particular, the questions in this thesis are addressed in three main parts:

In Manuscript 1, the process from precipitation to feed water is studied and the influence of organic surface on the isotope composition of adsorbed water is investigated. The following specific questions are addressed: (1) Does the surface effect differ among different organic materials? (2) Is the isotopic fractionation caused by surface effect independent of the isotopic composition of unconfined water? (3) Does the surface effect of organic matters influence the transfer of isotopic information from precipitation to feed water?

To this end, a large variety of organic materials were equilibrated via the gas phase with unconfined water of known isotopic composition to quantify the isotopic difference between adsorbed water and unconfined water. Additionally, we established a simple model to describe the surface effect and presented its versatility in an application case with environmental samples.

In Manuscript 2, the isotopic flow from diet to body water is investigated and the following questions are answered: (1) Is the $\delta^{18}O$ of milk influenced by feeding, ambient conditions, and sources of drinking water? (2) Does the seasonal variation mask the regional information of milk? (3) Can a newly developed mechanistic model well describe the seasonal isotopic variation of milk O in different farms?

To this end, the isotope composition of farms with different feeding strategies and sources of drinking water in southern Germany was measured throughout a year and an extended version of the Kohn model (which then is called Munich Kohn model or MK model) involving both feeding strategy and ambient conditions was established.

In Manuscript 3, the whole process from precipitation to hair O is investigated and the following specific questions are addressed: (1) How much does each O flux contribute to the seasonal isotopic variation of hair O? (2) What is the fractionation between body water and hair O? (3) Is hair a reliable tissue to judge the origin of cows?

To this end, the seasonal isotopic variation of hair O in a domestic suckler cow that underwent different ambient conditions, physiological states (lactation and non-lactation), keeping and feeding strategies was investigated. Additionally, the MK model was used to predict seasonal patterns of $\delta^{18}O$ in tail hair and explain its sources of variation.

2. General methods

The experiments covered two spatio-temporal scales differing in complexity. The short-term (hours) micro scale comprised laboratory experiments with rather homogenous material under controlled and constant ambient conditions. They were intended to investigate possible effect of organic surfaces on adsorbed water. The long-term (months to years) farm scale comprised experiments covering the full complexity of ambient conditions, farming activities and animal behavior. They were intended to study the

influence of these factors on the $\delta^{18}O$ of milk and hair. The farm-scale experiments, due to their complexity, were complemented by modelling experiments with a newly developed mechanistic model, the MK model.

2.1 Micro scale experiments

Dishes containing silage, hay, hemic litter, fibric litter, filter paper, bleached medical cotton, casein powder, and flour were all placed in closed chambers (glass exsiccator vessels with a volume of approximate 20 L with drying agent removed) to equilibrate with 200 mL unconfined water which was located at the bottom of the chambers. During equilibration, a recycling pump was turned on to ensure homogeneity within the airspace of the chamber. After 100 h of equilibration, samples and unconfined water were quickly removed from the chamber, placed in vials, sealed with a rubber stopper and wrapped with parafilm. The samples were then stored in an -18 °C freezer until water extraction by cryogenic vacuum distillation and measurement. The details of these experiments are shown in Manuscript 1.

2.2 Farm scale experiments

Two sets of farm based experiments were conducted: one investigated the $\delta^{18}O$ of milk and the other studied the $\delta^{18}O$ of hair (the overviews are presented in Table 2).

For the milk experiments, 28 farms in southern Germany, covering an area of about 80 km N-S and 370 km E-W, were investigated. The feed composition (grass silage or hay, maize silage, concentrates, and fresh grass either from pasture or cut) was obtained from interviews with the farmers. The feeding was grouped into three strategies depending on the main feed component: grass fed on pasture, stall feeding of cut grass and no grass (only silage or hay was fed in a stall). These strategies were called ‘pasture grass’, ‘cut

grass' and 'no grass' respectively for short. Each farm practiced one to three feeding strategies during the year. Well-mixed tank milk was sampled weekly on these farms and was immediately stored at −20 °C before thawing for $\delta^{18}O$ analyses. More details of sampling, site, and feeding strategies are described in Manuscript 2.

For the hair experiments, a suckler cow at the Grünschwaige Experimental Station was studied. The cow remained entirely at pasture during grazing seasons and was provided by a mixture of silage and hay during stall seasons. At the beginning and end of the grazing seasons, hair was collected from the tail switch of the cow and cut into segments of 1 cm length for isotopic measurements. More details are given in Manuscript 3.

Table 2: Overviews of farm based experiments.

Material	Year	Animal type	Sample number	Farm number	Feeding strategy
Milk	2005	Cows in lactation	608	28	Pasture grass Cut grass No grass
Hair	2000 to 2004	Suckler cow	191	1	Pasture grass No grass

2.3 Isotope analysis

Two methods were used to measure the isotope composition of water samples. (1) The $^{18}O/^{16}O$ in milk water was measured by an IsoPrime isotope ratio mass spectrometer that was interfaced to a multiflow equilibration unit (both GVI, Manchester, UK). (2) The other water samples were measured using Cavity Ring-Down Spectroscopy (Picarro, USA). Each sample was measured repeatedly (more than four injections) and the values of the last two measurements were averaged. For both methods, two laboratory water standards, derived from local deionized tap water by evaporation/condensation processes

and covering the range of the isotope compositions of the samples, were measured for possible drift correction and normalizing results to the VSMOW scale. The laboratory standards were previously calibrated against V-SMOW, V-GISP and V-SLAP by using the same analytical procedure as used in sample analysis.

The $\delta^{18}O$ of hair, was measured after packing the hair segments in silver cups (4 to 6 mm) by the pyrolysis method in a continuous flow system with an elemental analyzer (EURO EA 3028; Euro Vector, Milan, Italy) interfaced to an IsoPrime isotope ratio mass spectrometer (GV Instruments, Manchester, UK). Each sample was measured against a CO-reference gas calibrated against a secondary isotope standard (benzoic acid, IAEA-601).

O isotope data are presented as $\delta^{18}O$ (‰), where $\delta^{18}O = (R_{sample}/R_{standard} - 1)$, with R being the $^{18}O/^{16}O$ ratio in the sample and in the standard (V-SMOW), respectively.

2.4 Modelling

In principle, the isotopic information of precipitation enters into the animal through three ways (Fig. 2): (1) the precipitation enters into soil water and is, hence, up taken by grass (2) the adhering water in grass is ingested by cows, which is from the precipitation or dew that is usually from the rising of precipitation-derived soil water; (3) drinking water, generally represents the annual precipitation, is ingested by cows. By these three ways, the isotopic signal of precipitation is partly reflected in body water and, subsequently, in hair by some distortions of other input and output fluxes. This is well described by the MK model.

The main principle of the MK model is that the $\delta^{18}O$ of body water in a cow (δ_{bw}) on any day i is related to the body water composition of the previous day (i-1) and the input and output fluxes of O isotopes of day i:

$$(M_{inputO} \times \delta_{inputO} + M_{bw,i-1} \times \delta_{bw,i-1}) / (M_{inputO} + M_{bw,i-1}) = (M_{outputO} \times \delta_{outputO} + M_{bw,i} \times \delta_{bw,i}) / (M_{outputO} + M_{bw,i}) \quad \text{(Equation 2.1)}$$

where M_{inputO} and $M_{outputO}$ are the masses (mole) of the input and output fluxes of O; δ_{inputO} and $\delta_{outputO}$ are the O isotope compositions (‰) of the input (air O uptake, air water vapor into the lungs, chemically bound O in feed, feed moisture and drinking water) and output fluxes (CO_2 production, fecal water, milk water, orally and nasally exhaled respiratory water, O contributing to organic products, sweat water, transcutaneous water vapor, urea and urinary water).

Fecal water, milk water, sweat water and urinary water are derived from body water without obvious isotopic fractionation. Hence, their isotope composition is replaced by $\delta_{bw,i}$. The output fluxes subject to fractionation (CO_2, O in organic products, urea, respiratory and transcutaneous water vapor) are derived from $\delta_{bw,i} + \varepsilon$, where ε denotes the isotopic fractionation between an output flux and body water. Therefore $\delta_{bw,i}$ is solved:

$$\delta_{bw,i} = (M_{inputO} \times \delta_{inputO} + M_{bw,i-1} \times \delta_{bw,i-1} - M_{oral} \times \varepsilon_{oral} - M_{nasal} \times \varepsilon_{nasal} - M_{cutan} \times \varepsilon_{cutan} - M_{CO2} \times \varepsilon_{CO2} - M_p \times \varepsilon_p) / (M_{inputO} + M_{bw,i}) \quad \text{(Equation 2.2)}$$

where ε_{oral}, ε_{nasal}, ε_{cutan}, ε_{CO2} and ε_p are the fractionations of orally exhaled water, nasally exhaled water, transcutaneous vapor, CO_2 production and organic products relative to body water; M_{oral} M_{nasal}, M_{cutan}, M_{CO2}, and M_p are their masses (mole).

By knowing the δ_{bw} and the fractionation between hair O and body water, the isotope composition of hair can be calculated. The details of the MK model are described in Manuscripts 2 and 3 for milk and hair, respectively.

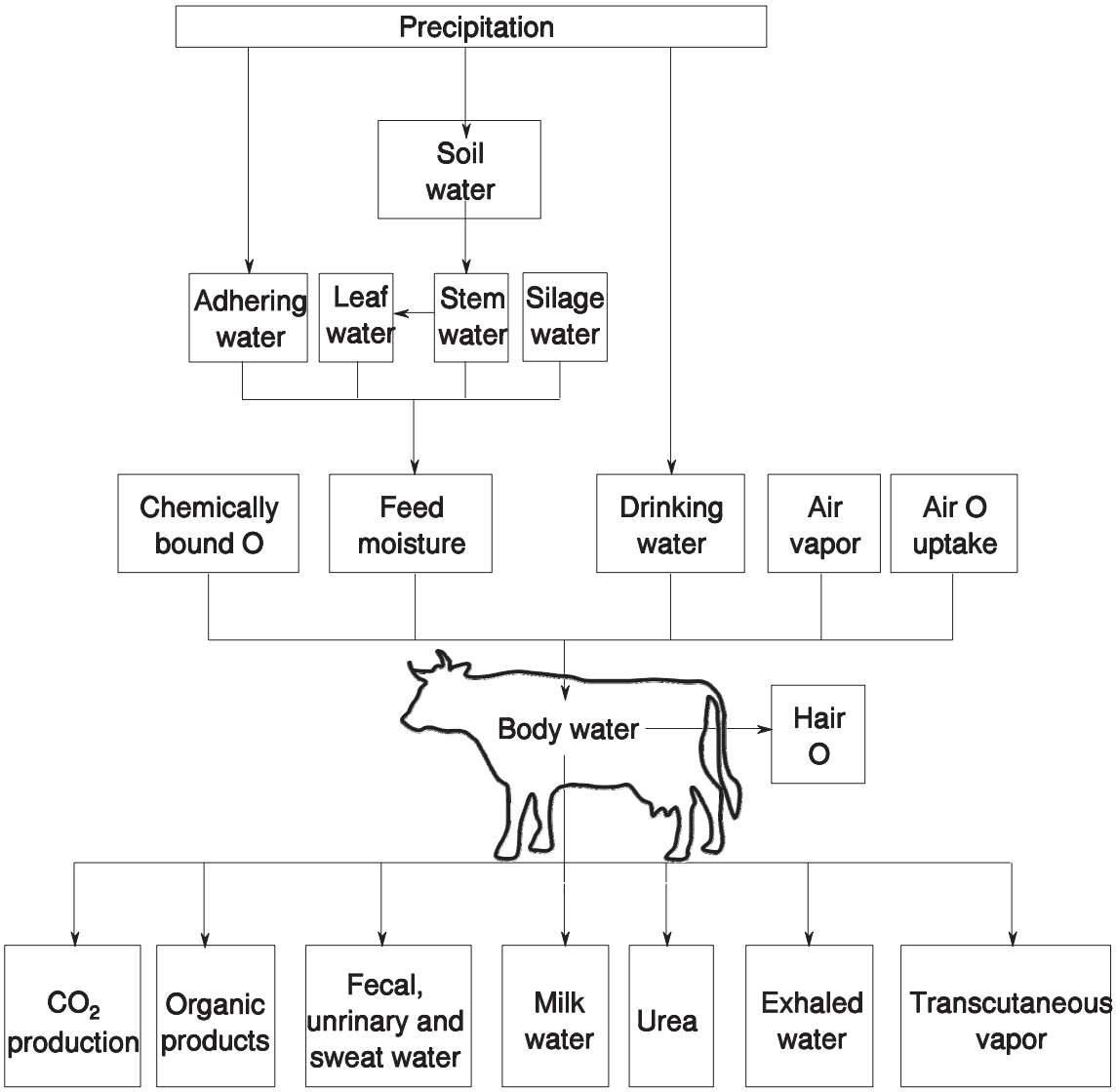

Fig. 2: Main oxygen flows from precipitation to body water and hair considered in this thesis.

The MK modelling requires estimating several water sources from ambient conditions, e.g., stem and leaf water results from precipitation either directly adhering to the outer surface or taken up via the soil; the silage and hay water equilibrates with air humidity

under ambient conditions. In this way ambient conditions influence the water taken up by the animal. The estimation of the isotopic relation of these water sources from ambient conditions by applying accepted isotopic principles is only true if these water sources behave like unconfined water. This may not be true for water in close contact to (organic) surfaces. If this is not the case, soil water could not be directly estimated from precipitation, stem water could not directly be predicted from soil water and silage water change on the feed bunk could not be directly estimated from air humidity. Hence, the farm-scale MK model had to be complemented by another model on the very different micro scale, the surface effect model.

The main principle of the surface effect model is the equilibration between atmosphere and outer layer water (unconfined water) in organic matters and a constant fractionation between outer layer water and inner layer water. If the thickness of inner layer is constant, the apparent isotopic fractionation between total water and outer layer water should increase with decreasing water content of the organic material.

The apparent fractionation between the total water and the unconfined water $\varepsilon_{T/U}$ can be estimated from the ratio between R_I, the volumetric ratio of solid:water associated with the layer that is influenced by the surface, and R_T, the volumetric solid:water ratio of total adsorbed water:

$$\varepsilon_{T/U} = \varepsilon_{S/U} \times R_T/R_I \qquad \text{(Equation 2.3)}$$

A detailed derivation of this model can be found in Manuscript 1.

2.5 Statistics

Data are usually presented as mean value ± standard deviation. Simple linear and quadratic regressions were used to analyze the relations between two parameters.

Multiple regression was used to quantify the multiple influences of parameters. Paired t-test was used to compare the difference between average modelled and measured values. The root mean squared error (RMSE) was used to quantify mean deviations between prediction and measurement. Significance, if not explicitly stated, always refers to $p < 0.05$.

3. Summaries of manuscripts and contributions of the authors

This thesis contains three manuscripts. Their publication status, the abstracts and the contributions of the authors are given in the following.

3.1 Manuscript 1: ^{2}H and ^{18}O depletion of water close to organic surfaces

Guo Chen, Karl Auerswald, and Hans Schnyder

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Hydrophilic surfaces influence the structure of water close to them and may thus affect the isotope composition of water. Such an effect should be relevant and detectable for materials with large surface areas and low water contents. The relationship between the volumetric solid:water ratio and the isotopic fractionation between adsorbed water and unconfined water was investigated for the materials silage, hay, organic soil (litter), filter paper, cotton, casein and flour. Each of these materials was equilibrated via the gas phase with unconfined water of known isotopic composition to quantify the isotopic difference between adsorbed water and unconfined water. Across all materials, isotopic fractionation was significant ($p < 0.05$) and negative (on average -0.91 ± 0.22 ‰ for $^{18/16}O$ and -20.6 ± 2.4 ‰ for $^{2/1}H$ at an average solid:water ratio of 0.9). The observed isotopic fractionation was not caused by solutes, volatiles or old water because the

fractionation did not disappear for washed or oven dried silage, the isotopic fractionation was also found in filter paper and cotton, and the fractionation was independent of the isotopic composition of the unconfined water. Isotopic fractionation became linearly more negative with increasing volumetric solid:water ratio and even exceeded -4 ‰ for $^{18/16}$O and -44 ‰ for $^{2/1}$H. This fractionation behavior could be modeled by assuming two water layers: a thin layer that is in direct contact and influenced by the surface of the solid and a second layer of varying thickness depending on the total moisture content that is in equilibrium with the surrounding vapor. When the model was applied to soil water under grassland, the soil water extracted from 7 cm and 20 cm depth was significantly closer to local meteoric water than without correction for the surface effect. This study has major implications for the interpretation of the isotopic composition of water extracted from organic matter, especially when the volumetric solid:water ratio is larger than 0.5 or for processes occurring at the solid-water interface.

Guo Chen and Karl Auerswald designed the experiments, analyzed the data and developed the model. Guo Chen carried out the experiments and wrote a first draft. All authors developed the manuscript and approved the final version.

3.2 Manuscript 2: Ambient conditions and feeding strategy influence $\delta^{18}O$ of milk water in cows (*Bos taurus*)

Guo Chen, Rudi Schäufele, Karl Auerswald

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There are increasing concerns by consumers regarding agricultural product traceability and authenticity. Oxygen isotope composition ($\delta^{18}O$) has been used in this context and is

based on the relationship between $\delta^{18}O$ of animal products and that of annual precipitation. However, in dairy products this relationship is affected by the seasonality of $\delta^{18}O$ in milk water which in turn depends on the feeding system used. We measured 608 milk samples from 28 farms with various feeding strategies in southern Germany throughout the year to investigate the influences of ambient conditions, drinking water source and feeding strategies on seasonal variation of $\delta^{18}O$ in milk water (δ_{milk}). The mechanistic Munich-Kohn model reflecting these influences and body water turnover predicted the seasonal and farm-specific variation of δ_{milk} well. The relationship between $\delta^{18}O$ of precipitation and δ_{milk} varied in different feeding strategies. The interplay of ambient conditions and feeding strategy on δ_{milk} should thus be carefully considered when identifying the origin of milk.

The isotope measurements were supervised by Rudi Schäufele. The data were analyzed by Guo Chen. The model was set up by Guo Chen and Karl Auerswald and parametrized by Guo Chen. Guo Chen wrote the first draft. All authors further developed the manuscript.

3.3 Manuscript 3: Model explanation of the seasonal variation of $\delta^{18}O$ in cow (*Bos taurus*) hair under temperate conditions

Guo Chen, Hans Schnyder, Karl Auerswald

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Oxygen isotopes ($\delta^{18}O$) in animal and human tissues are expected to be good recorders of geographical origin and migration histories. However, seasonal variation of $\delta^{18}O$ may diminish the origin information in the tissues. Here the seasonality of $\delta^{18}O$ in tail hair was

investigated in a domestic suckler cow (*Bos taurus*) that underwent different ambient conditions, physiological states, keeping and feeding during five years. A detailed mechanistic model was built to explain this variation. The measured $\delta^{18}O$ in hair significantly related ($p < 0.05$) to the $\delta^{18}O$ in meteoric water in a regression analysis. Modelling suggested that this relation was only partly derived from the direct influence of feed moisture. Ambient conditions (temperature, moisture) also affected the animal itself (drinking water demand, transcutaneous vapor etc.). The clear temporal variation thus resulted from complex interactions with multiple influences. The twofold influence of ambient conditions via the feed and via the animal itself is advantageous for tracing the geographic origin because $\delta^{18}O$ is then less influenced by variations in moisture uptake; however, it is unfavorable for indicating the production system, e.g. to distinguish between milk produced from fresh grass or from silage. The model is versatile but needs testing under a wider range of conditions.

The data were taken from an earlier publication on a different topic. Guo Chen and Karl Auerswald set up the model. Guo Chen analyzed the data and wrote the first draft. Karl Auerswald put forward the idea of the paper and revised the manuscript. Hans Schnyder reviewed the manuscript.

4. Main findings

Before the general discussion of this thesis, I will briefly outline below the primary results and findings from the manuscripts contained in this thesis.

The first manuscript investigated the influence of hydrophilic surfaces of organic matters on the isotope composition of water close to them. The volumetric solid:water ratio was significantly ($P < 0.05$) and negatively related to the isotopic fractionation between adsorbed water and unconfined water. The fractionation of $\delta^{18}O$ and $\delta^{2}H$ was negative

and significant ($P < 0.05$) for all materials, except for $^{18}/^{16}O$ with filter paper and cotton and for $^{2/1}H$ in a few samples of cotton. This significant relation was also found for all the unconfined water with different isotope composition and the fractionation was independent of the isotope composition of unconfined water, indicating that the fractionation was not caused by insufficient time for equilibration. The relationship between apparent isotopic fractionations of washed, oven dried and fresh silage and solid:water ratio followed the same line and the areas overlapped each other for these three types of silage. This suggested that the observed isotopic fractionation was also not caused by solutes and volatiles. Additionally, predicted isotopic fractionation by incomplete extraction based on a Rayleigh fractionation fell far apart from the observed isotopic fractionation, suggesting that the fractionation was not caused by the error of incomplete extraction. The variation of apparent isotopic fractionation with water content was well described by a simple, easy to apply two-layer model. The model can be successfully applied to soil water under grassland in winter: the corrected soil unconfined water at 7 cm and 20 cm depth by model was significantly closer to local meteoric water than without correction for the surface effect.

In the second manuscript, a seasonality of the δ_{milk} was found in the farm in southern Germany. The values ranged from -2 to -10 ‰ with higher values in summer than in winter. The highest value appeared for the pasture grass strategy in summer, while the lowest values were found for the strategy of no grass in winter. The quadratic regressions between δ_{milk} and day of year for fresh and cut grass strategies were both significantly different ($P < 0.05$) from that for the no-grass strategy. $\delta^{18}O$ of monthly precipitation was significantly ($P < 0.05$) and positively related to that of milk in all strategies. Pasture grass and cut grass strategies exhibited almost the same linear regressions, while the no-

grass strategy caused lower values. The feeding components also influenced the δ_{milk}: high grass content or low silage or hay content yielded enriched value. Additionally, the sources of drinking water influenced the δ_{milk}: the values in lake sourced farms and paleo-water sourced farm are more enriched and depleted than in other farms, respectively. The MK model estimated the seasonal variation of δ_{milk} generally well, however, some uncertainties of the model were created by animal, silage, farm and precipitation.

In the third manuscript, the seasonality of δ_{hair} in a domestic suckler cow that underwent grazing seasons and stall seasons was investigated. During the grazing season the hair was more enriched than in the stall season, even when temperature, relative humidity or vapor pressure deficit were identical. Temperature and relative humidity were significantly positively and negatively related to δ_{hair}, respectively. The measured δ_{hair} was also significantly related ($P < 0.05$) to the $\delta^{18}O$ in precipitation in a regression analysis, however, this relation was only partly derived from the direct influence of feed moisture. Ambient conditions (temperature, moisture) did not only influence the isotopic signal of precipitation but also simultaneously affected the animal itself (drinking water demand, transcutaneous vapor etc.). By modeling analysis, three parameters, namely drinking water, feed internal water and ambient conditions influencing the animal, were the main sources of variation in δ_{hair}. The mechanistic MK model explained well the variation between seasons and within seasons.

5. General discussion

5.1 Transfer of isotopic information from precipitation to hair

The isotopic information of precipitation may be distorted until it enters into the hair, which happens during three steps: (1) from precipitation to diet (in the forms of grass

water, adhering water, silage or hay water and drinking water); (2) from diet to body water; (3) from body water to hair (Fig. 2).

5.1.1 From precipitation to diet

Grass water: The isotopic signal of precipitation enters into the soil water and, subsequently, it is transferred to the stem water (Brooks et al., 2010). This principal flow of isotopic information was corroborated by significant regression between stem water (or soil water) and precipitation based on the observation from 2006 to 2012 in Grünschwaige Experimental Station (Fig. S2 in manuscript 3) but this relation was surprisingly weak ($R^2 = 0.20$). Given that the step from precipitation to stem water is a rather direct step and many more steps like plant transpiration and mixing of feed components follow, a close causal relation between precipitation and body water or body tissues cannot exist despite the correlations that were reported in several publications (Chesson et al., 2010; Ehleringer et al., 2008) and found here for milk and hair. This already indicates that the relations between precipitation and body water or body tissues must be due to indirect effects.

The reasons why soil water and subsequently stem water differ from precipitation are manifold. Exploring them in detail is beyond the scope of this thesis. A main reason is that soil water preserves the information of previous precipitation (Ma and Song, 2016) and thus causes a delay of several months between precipitation and soil water. This was also the case for the data shown in Fig. S2 in manuscript 3. For these data, $\delta^{18}O$ in precipitation peaked in July/August, while $\delta^{18}O$ in stem water peaked in September/October. More effects than mixing of new and old soil water may occur like preferential flow, in which the precipitation is channeled through more permeable pathways (Gazis and Feng, 2004; Sukhija et al., 2003). The isotopic signal of the soil

water thus even varies on a small scale due to the fingered flow (Thomas et al., 2013). Furthermore it will depend on the depth of water uptake by the plants (Yang et al., 2011). During dry periods they may use deep and old water sources while during wet periods they may use water from the top layers of the soil that are closer connected to recent precipitation than deep layers (Dawson and Pate, 1996).

Contrary to what may be expected on first glance, the difference between stem water and precipitation unlikely results from the surface effect, although the surface effect was second highest in soil among the materials relevant for this thesis (Fig. 3). A larger surface effect could only be found in hay, which had the lowest water content among all materials. The reason for this apparent contradiction is that the surface effect only appears in tightly bound water, which is only captured if all the water is extracted. This tightly bound water is unavailable to plants, which take up only mobile water that should be unaffected by the surface effect because it is what was called unconfined water in manuscript 1.

The surface effect is also ignorable for stem water because of the high water content (75 to 85 %) that creates only a small ^{18}O fractionation (-0.20 ‰ to -0.09 ‰) and again because the confined part of the stem water would not move to the leaf. Hence only the intake of stem water (or pseudo-stem water) itself by the grazer will be affected. This intake contributes little to the total water intake from grass because grazers preferentially forage leaves (Pinchak et al., 1991).

The stem water, then, transfers to leaf and the water signal changes a lot due to the evaporation of leaf water, which is influenced by ambient conditions (temperature and humidity) and plant physiology (Cernusak et al., 2016; Liu et al., 2016). The leaf water fluctuates considerably even within one day due in response to the fluctuation of ambient

conditions (Fig S5 in Manuscript 3). This is especially true under extremely changing weather (e.g. a short storm) (Cernusak et al., 2002; Jones et al., 2010; West et al., 2006). Hence, the leaf water did not reflect the isotopic information of precipitation (Fig. S2 in manuscript 3) but contributed about 29 % to the total water intake during the growing season (with the largest share of the remaining intake being drinking water, which also does not carry the seasonal variation of precipitation).

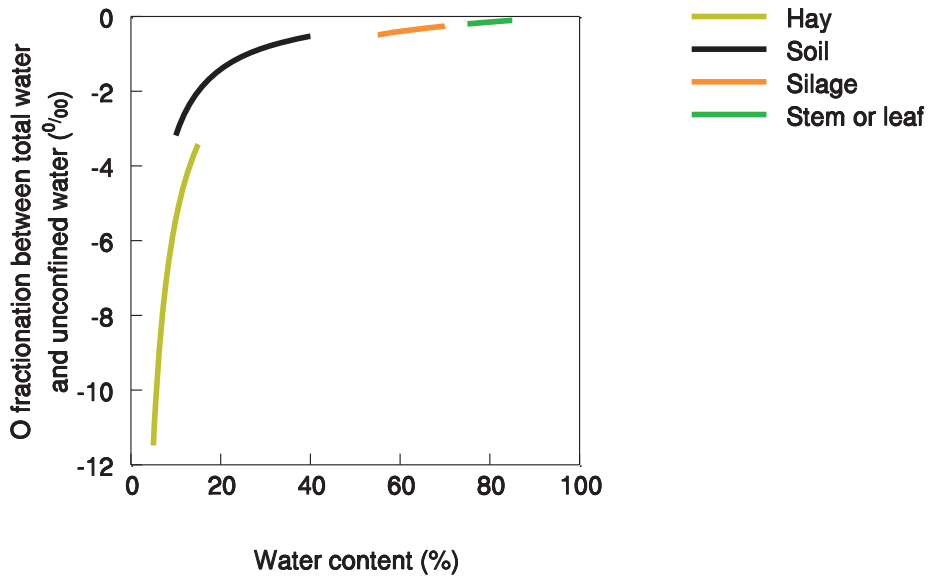

Fig. 3: Modelled fractionations between total water and unconfined water in hay, soil, silage, and stem or leaf within their typical ranges of water contents (5 to 15 %, 10 to 40 %, 55 to 70 % and 75 to 85 % based on fresh matter, respectively). The surface effect of soil water is shifted compared to the relation of the other materials because in manuscript 1 it was assumed that sand, with small surface area, can be excluded in the calculation of the surface effect of soil. Thus, the water layer above the other surfaces of soil is thicker than what would be expected from the average water content of the soil. This causes a smaller surface effect at a given water content.

Adhering water to grass: The MK model assumes that the isotope composition of adhering water is equal to that of precipitation, because both precipitation and adhering water equilibrate with the local vapor at similar temperature and 100 % humidity (Kim and Lee, 2011). Hence this adhering water is the only water that could directly transfer the isotopic signal of precipitation to the grazer. However, adhering water contributed only 2 % of the O total input and can hardly explain the correlation between body water and precipitation. Furthermore, the adhering water is also influenced by the ambient conditions and an hourly variation has been reported in dew (about 0.4 ‰ from 9:00 to 12:00) (Zhang et al., 2009). Especially evaporation of adhering water after the end of a rain event or after sunrise in the case of dew will cause an enrichment of ^{18}O similar to leaf water.

Silage or hay water: When silage is produced by drying grass on the soil surface after cutting, the water may exchange with the rising moisture from precipitation-derived soil water (Sun et al., 2014). This should imprint the (delayed) information of precipitation on silage water. However, the silage is stored in silo without contact to air or other water sources (Bernardes and do Rego, 2014) and fed several months later. Thus silage also cannot carry the isotopic characteristics of precipitation at the time of feeding. In addition, the surface effect causes a small deviation (within 1 ‰) between unconfined (precipitation) water and silage water (Fig. 3).

The only pathway how the isotopic characteristics of recent precipitation could enter silage (or hay) is by exchange with air humidity at the silo front or while it is on the feed bunk (Sun et al., 2014) because during rain, air humidity should be in isotopic equilibrium with the rain (Gat, 1996). This, however, would already be an indirect effect

because the silage (or hay) usually is not exposed to the rain itself. In consequence, the evident correlation between body water and precipitation is practically entirely shaped by indirect effects. The indirect effect via silage (or hay) is the least important. Most of the indirect effects occur at the animal level (see next chapter).

Drinking water: It is usually assumed that the isotope composition of drinking water is close to that of mean precipitation (Bowen et al., 2007). In consequence it cannot cause the relation between the seasonal changes in precipitation and body water. Even for the annual mean, this assumption was surprisingly often not true within the research area (Central Europe) because tap water of four out of the 29 farms studied in this thesis was either derived from paleo water or from lake water. For Germany it was estimated that even 9% of the tap water is from lakes and dams, 6% is bank storage water, 10% is groundwater artificially recharged with surface water and 1% is river water (Wohlrab et al., 1992). Thus in total 25% is not groundwater but surface water that has undergone isotopic change due to evaporation. Even neighboring farms receiving similar precipitation may differ in drinking water when supplied by different water works. Also in areas where fractured bedrock aquifers dominate, lake water or dam water is frequently used and may be pumped over distances in the range of several 100 km (280 km in the case of Lake Constance water (King and Volker, 2004). In cases where surface water is used, the tap water is above mean local precipitation because it is enriched in $\delta^{18}O$ due to lake evaporation (Kennedy et al., 2011; Zhao et al., 2017). In mountain areas, the sources of drinking water may be even more divers: in stall seasons, cows are usually supplied by tap water, while in grazing seasons, stream water or local spring water is afforded, which will exhibit some seasonal variation although is does not exactly reflect the seasonal

variation in precipitation because of the delay and mixing effects during the soil passage (Jencso et al., 2010).

5.1.2 From diet to body water

According to the above analysis, the isotope compositions of grass, silage and hay water can hardly reflect the isotopic information of precipitation. However, their amounts influence the proportion of the drinking water, which differs considerably in isotopic composition especially compared to grass. When the hay or silage is fed, more drinking water is required, so the isotope composition of the water taken in by the animal is close to that of drinking water. When grass is fed, the isotope composition of the intake water is far away from drinking water due to the influence of enriched grass water. Thus, a seasonal variation in the isotopic composition of the intake water results. This variation resembles the seasonal variation in precipitation although it is practically not influenced by precipitation.

However, a seasonal variation in milk water also occurred in cases where constant feed was supplied to the cows because of the higher transcutaneous water losses at high ambient temperatures that cause enrichment of the body water. The strong effect of the transcutaneous water losses on the isotopic composition of body water can be nicely illustrated by the MK model. When body weight is increased and thus the ratio of surface area to body water mass decreases, the enrichment of body water compared to drinking water will decrease even under otherwise completely identical conditions (Fig. 4). However, systematic study of this enrichment in different species is still needed.

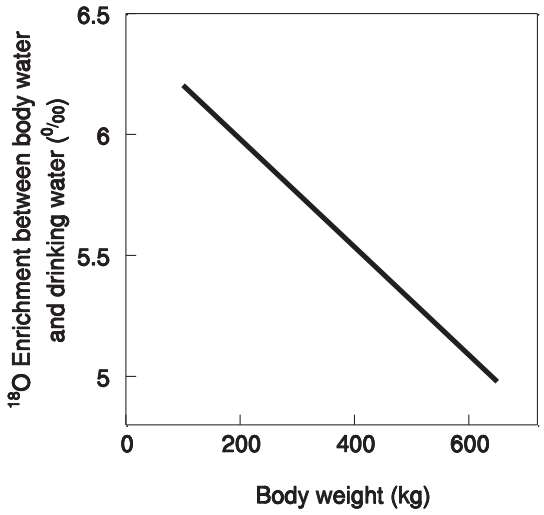

Fig. 4: Modelled relationship between body weight and average daily $\delta^{18}O$ of body water for cows in Grünschwaige Experimental Station when the body weight is set to different values in the MK model.

5.1.3 From body water to hair

The hair O is more enriched than body water (Kornexl et al., 1997; Podlesak et al., 2008), which is due to the positive fractionation between carbonyl O and water. Ehleringer et al. (2008), Bowen et al. (2009) and O'Grady et al. (2012) built a model to explain the transfer of isotopic signal from body water to hair in humans, nonhuman primates and woodrats. They propose that $\delta^{18}O$ in hair is derived from isotopic exchange of O in C terminus with gut water during hydrolysis of dietary protein. However, the model does not consider the O in the R-groups of some amino acids (e.g. the O in hydroxyl groups in serine, threonine and tyrosine; the O in sulphur-containing amino acids like cysteic acid; the O in carboxyl groups and amide groups of the lateral chains of amino acids like aspartic acid, glutamic acid, asparagine and glutamine). It may not be justified to ignore these amino acids, because they make up about 45 % of the total amino acids in the

keratin (Popescu and Hocker, 2007). Also, the isotopic fractionation between hair O and gut water in the model was never actually measured in animals, it was either from the fitting of model or from the fractionation effects between media water and organic matter observed in microbial spore cell walls during non-log-phase growth (Ehleringer et al., 2008; Kreuzer-Martin et al., 2005).

Furthermore, the model may not reflect the situation in ruminants. Cows have a four-compartment stomach, which involves water cycling, absorption and remixing, which makes the estimation of gut water difficult. Additionally, ruminal microorganisms contribute considerably to protein synthesis. In particular they produce the essential amino acid threonine, which carries nonexchangeable O in the R-group (Purser and Buechler, 1966).

For simplicity and given the lack in scientific knowledge, a constant fractionation between hair O and body water was used and the best fitted value was 14 ‰ (manuscript 3). However, it has to be noted that this fractionation seems to vary between and even within species in different studies (O'Brien and Wooller, 2007; Podlesak et al., 2008). Whether these differences are true or caused by difference in methodology is unknown. Especially the hair treatment before measurement may contribute to the uncertainty of the measurement of hair O (Bowen et al., 2005a) and, thus, to the uncertainty of fractionation between body water and hair O. The process of storing and transfer of samples after washing and subsequent drying may introduce error because water absorption from air humidity can add up to 10 % of non-biological O within six hours (Bowen et al., 2005a; Fischer, 2006). It is also reported that washed hair dried at different temperatures can differ by 2 ‰ (Fischer, 2006). Standardized pre-treatment methods are necessary in order to obtain reliable and comparable results.

5.2 Body water pool and turnover

Pool size and turnover are crucial for modeling the isotopic flow. In the MK model, the body water pool was assumed to be well mixed, which was in accordance with the results from Abeni et al. (2005) who found similar O isotope composition in plasma, urine and milk water in cows. Although a slight difference (1 ‰) between tissue water and body water was found in rats (Kreuzer et al., 2012) and medium water was also different from cell water in microbes with high metabolic rates, no evidence is available to suggest that such gradients exist in cells with low metabolic rates (Kreuzer-Martin et al., 2005) and in domestic animals, which justifies the simplifying assumption of a well mixed body water pool in the MK model. However, theoretical considerations (Langmuir, 1908) on turnover suggest that water turnover should differ in different organs depending on the ratio between the amount of water present in an organ and the flow rate of water through this organ similar to the differences in half-life of $\delta^{15}N$ and $\delta^{13}C$ in different organs (Bahar et al., 2009; Braun et al., 2013; Pearson et al., 2003). The turnover of water in different organs and tissues is necessary to be investigated in further work.

Based on the assumption of one body water pool, the average half-life of the water in cows can be simply calculated by assuming the feed water and drinking water constantly mixes with body water every day. On average of all cows modelled in this thesis, half-life of body water was 5 d. However, in reality the half-life of cows can hardly be stable because the amount ratio of water intake to body water varies (from 0.1 to 0.25 for cows in the 28 commercial farms according to the MK model), producing half-lives of 3.1 to 7.3 d. This is in accordance with the range of half-life (2.9 to 7.5 d) in cows given by Abeni et al. (2015), suggesting the feasibility of the MK model in this respect.

5.3 Application

This thesis covered three tools of potentially large future relevance: the use $\delta^{18}O$ in body water and tissues, the application of the MK model, and the (modelling of the) surface effect.

$\delta^{18}O$ in body water and tissues:

The $\delta^{18}O$ turned out to be dominantly controlled by the ambient conditions an animal is exposed to. This makes $\delta^{18}O$ a suitable tool for origin tracing because the impacts of feeding strategy and other confounding influences is rather small. This finding agrees with the main present use of $\delta^{18}O$ in animal tissues and products (Bowen et al., 2005c; Hobson et al., 2004). However, the strong seasonal variation of ambient conditions – in combination with the seasonal variation of main feed sources – makes it indispensable to account for season. Furthermore, this conclusion is only true for bulked products like tank milk or wool, which average over animals and/or time. The smaller the temporal scale and the number of animals become, the more $\delta^{18}O$ will depend on other influences like the variation of $\delta^{18}O$ between rain events or the influence of body weight or animal behavior. This implies that $\delta^{18}O$ can become a valuable tool in studies of animal physiology and behavior but such studies need to be assisted by the MK model (see below).

MK Model:

Given the high complexity of influences and feedback mechanisms on $\delta^{18}O$ in body water and tissues, simple cause-effect relations do not exist. In this situation, the MK model is a useful tool for predicting the resulting effects in the fields of both, characterization of production systems and origin tracing. These predictions can be seen as quantitative hypotheses that then can be examined in animal experiments. During the setup of such

animal experiments, the MK model allows identification of those parameters that are essential to be controlled or measured.

In terms of production systems, the MK model can be potentially used to estimate the impact of ambient conditions that cows undergo. For example, heat stress is of frequent concern by farmers because it decreases feed intake and milk production (West et al., 2003). Animal respond to heat stress by panting, sweating and increasing transcutaneous water losses. All three pathways differ considerably in their effect on $\delta^{18}O$ in body water. Especially sweating and transcutaneous water losses are usually considered together (Thompson et al., 2011). Measurements of $\delta^{18}O$ in body water could be a tool to distinguish between both pathways and the MK model could help in setting up appropriate experiments. The proportion of stems and leaves ingested during grazing are difficult to record and largely deviating estimates exist in literature (Durham and Kothmann, 1977). The large contrast in $\delta^{18}O$ between stem and leaf water may be used to quantify this ratio and its controls but this has to be assisted by modelling because difference of $\delta^{18}O$ in ingested feed caused by difference in the stem-to-leaf ratio will only partly appear in body water due to processes at the animal level. Another application of the MK model may also be to evaluate the effect of diseases. They may change body temperature and feed intake (Benzaquen et al., 2007; Ostergaard and Grohn, 1999) and thus should be traceable by $\delta^{18}O$ in body water.

In terms of origin tracing, many parameters (e.g. ambient conditions, feeding time, and feed components) potentially influence the $\delta^{18}O$ of animal tissues or products that hardly can be examined in all combinations by animal experiments. The MK model may be used to disentangle the convoluted impact of different parameters and to identify those parameters that should be carefully considered in origin tracing.

Surface Effect:

This study offers for the first time an explanation and a prediction equation for what has been described as "two water worlds" (Evaristo et al., 2015). Although both, the explanation and the prediction equation proposed here, need to be confirmed in other studies and deserve refinement, they allow an assessment of which water world is relevant for a specific question. This distinction is of high relevance because usually all water has to be extracted from a sample in order to avoid Rayleigh fractionation while only one water world may be relevant. It may be the tightly bound water that governs processes directly at the surface, or it may be the mobile, almost unconfined water, or it may be both (as for the ingestion of silage by animals).

6. Conclusions

The transfer of O isotopic information from precipitation to animal tissues or products is a complex process influenced by plant physiology, feeding strategy, ambient conditions and animal physiology. These influences cause large inter-seasonal and intra-seasonal variations in body water. A newly developed model, the MK model, explained the isotopic variation in body water or animal products well. The inter-seasonal and intra-seasonal variations in body water should be deliberately taken into account when using $\delta^{18}O$ for geographical origin assignment. While wild animals and their feed are exposed to the same ambient conditions and thus carry the same geographic information, the feed, drinking water and the ambient conditions of domestic animals are strongly modified by the farmer. This can additionally distort the geographic information. The MK model is a helpful tool for separating and quantifying the geographic, inter-seasonal, intra-seasonal and human influences on $\delta^{18}O$ on body water of domestic animals.

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Appendix

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^{2}H and ^{18}O depletion of water close to organic surfaces

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Abstract. Hydrophilic surfaces influence the structure of water close to them and may thus affect the isotope composition of water. Such an effect should be relevant and detectable for materials with large surface areas and low water contents. The relationship between the volumetric solid : water ratio and the isotopic fractionation between adsorbed water and unconfined water was investigated for the materials silage, hay, organic soil (litter), filter paper, cotton, casein and flour. Each of these materials was equilibrated via the gas phase with unconfined water of known isotopic composition to quantify the isotopic difference between adsorbed water and unconfined water. Across all materials, isotopic fractionation was significant ($p<0.05$) and negative (on average -0.91 ± 0.22 ‰ for $^{18/16}O$ and -20.6 ± 2.4 ‰ for $^{2/1}H$ at an average solid : water ratio of 0.9). The observed isotopic fractionation was not caused by solutes, volatiles or old water because the fractionation did not disappear for washed or oven-dried silage, the isotopic fractionation was also found in filter paper and cotton, and the fractionation was independent of the isotopic composition of the unconfined water. Isotopic fractionation became linearly more negative with increasing volumetric solid : water ratio and even exceeded -4 ‰ for $^{18/16}O$ and -44 ‰ for $^{2/1}H$. This fractionation behaviour could be modelled by assuming two water layers: a thin layer that is in direct contact and influenced by the surface of the solid and a second layer of varying thickness depending on the total moisture content that is in equilibrium with the surrounding vapour. When we applied the model to soil water under grassland, the soil water extracted from 7 and 20 cm depth was significantly closer to local meteoric water than without correction for the surface effect. This study has major implications for the interpretation of the isotopic composition of water extracted from organic matter, especially when the volumetric solid : water ratio is larger than 0.5 or for processes occurring at the solid–water interface.

1 Introduction

The $^{18/16}O$ and $^{2/1}H$ isotope composition of water reflects climate and many processes within the water cycle (Bowen, 2010; Gat, 1996). Changes in the isotope composition of water can either result from the mixing of water with differing isotopic composition or from the change in isotopic composition by fractionation, especially between vapour and liquid. The vapour/liquid fractionation is not only affected by temperature but also by ion hydration (Kakiuchi, 2007). In aqueous solutions, ions change the activities of the isotopologues of water (H_2O, HDO and $H_2^{18}O$) due to their hydration. This, in turn, causes the isotopic fractionation between aqueous solutions and water vapour to differ from the fractionation between pure water and vapour (Kakiuchi, 2007; Stewart and Friedman, 1975). Similar to salt, the surface of hydrophilic materials also interacts with water molecules creating a two-dimensional ice-like water layer near the surface and a three-dimensional liquid layer far from the surface (Asay and Kim, 2005; Miranda et al., 1998). Additionally, adsorption may cause an energetic difference between water molecules at the surface of solids and the bulk water molecules (Richard et al., 2007). These structural and energetic differences may cause a difference in isotopic composition between these two layers of water. If existent, such a surface effect should be strongest in materials with large specific surface area and with low water content. There are some indirect hints from studies of plant water uptake from soil, which show that mobile water differs isotopically from immobile water (Brooks et al., 2010; Evaristo et al., 2015; Tang and Feng, 2001) but to the best of our knowledge, such a surface effect has only been directly studied for clay (Oerter et al., 2014) and silica surfaces (Richard et al., 2007). It is not known how large the effect is for organic matter, which is associated with practically all mineral surfaces in the critical zone or forms major

constituents of other surfaces in the biosphere (Chorover et al., 2007; Nordt et al., 2012; Vazquez-Ortega et al., 2014).

A surface effect may be detected by establishing equilibrium between water adsorbed to a material and air vapour created by unconfined water with known isotope composition in a closed chamber. If there is no surface effect, then the $^{18/16}O$ and $^{2/1}H$ isotope composition of the adsorbed water and unconfined water should be identical after equilibration. This is because the isotope composition of water under steady conditions is determined by the isotope composition of the water vapour, air humidity, equilibrium fractionation and kinetic fractionation (Helliker and Griffiths, 2007; Welhan and Fritz, 1977). All of these parameters are identical for adsorbed water and unconfined water when they both share the same atmosphere in a closed chamber for a sufficiently long time.

We examined the hypothesis that the surfaces of organic materials influence the isotopic composition of adsorbed water and we choose materials of broad relevance. Silage, the product of anaerobic fermentation of fresh forage, is an important feedstuff, which also delivers water to the animal and thus influences body water composition (Kohn, 1996; Soest, 1994; Wilkinson, 2005) and animal products like milk. Hay has particularly low water content. Organic horizons at the soil surface provide the interface through which most vapour and water flows have to pass (Haverd and Cuntz, 2010). More materials like filter paper, cotton, protein powder and wheat flour were included to identify whether the chemical identity causes or influences the effect. Finally we had to exclude that the effect resulted from artefacts like old water or volatiles and solutes interfering with the isotope measurements (Martín-Gómez et al., 2015; Schmidt et al., 2012; Schultz et al., 2011; West et al., 2011). Silage, which is likely a source of volatiles and solutes in rather large amounts (e.g. lactic acid, acetic acid, propionic acid, ethanol and propanol; Porter and Murray, 2001), was also pretreated by washing and heating to remove potentially interfering substances. Water of contrasting isotope composition was used to identify old water. Finally, we derived a simple prediction model for the effect and demonstrated its versatility in an application case with environmental samples.

2 Materials and methods

We performed three equilibration experiments. Each equilibration experiment involved the exposure of samples to water vapour, which originated from unconfined water, followed by cryogenic water extraction from samples and isotope composition measurement. We use $\delta^{18/16}O$ and $\delta^{2/1}H$ to describe the isotope composition of oxygen ($^{18/16}O$) and hydrogen ($^{2/1}H$) in water (with $\delta^{18/16}O$ or $\delta^{2/1}H = R_{sample}/R_{standard} - 1$, where R_{sample} and $R_{standard}$ denote the ratio of the abundances of heavy and light isotopes in samples following the international SMOW standard).

2.1 Preparation of samples

The materials comprised fresh silage, oven-dried silage, washed silage, hay, fibric and hemic litter, filter paper, cotton, casein and wheat flour. Silage was also oven dried to remove all volatiles and washed to remove all solutes. Fibric litter is slightly decomposed organic material on top of the mineral soil derived from plant litter, thus more decomposed than silage but partly still resembling the structure of plant organs. Hemic litter is strongly decomposed organic material of low fiber content, which has lost the structure of the plant litter but contains dark brown soluble substances that dye the water extract (Schoeneberger et al., 2012). More pure materials were included to identify whether the chemical identity causes or influences the effect. We used filter paper and cotton to represent pure cellulose, the most common plant material, commercial wheat flour to represent less pure carbohydrates including branched carbohydrates and commercial casein powder to represent proteins.

The silage and hay were obtained from a farm near Freising and cut into pieces (4 to 8 cm). The silage was stored in a −18 °C deep freezer while the hay was kept in a dark and dry place before use. The hemic and fibric horizons were gathered from a conifer forest near Freising (Germany) from a Haplic Podzol (according to IUSS Working Group WRB, 2014) area and stored in airtight bags in a refrigerator until use. In order to create a relative wide range of water content, and half of the litter samples were oven dried (16 h for 100 °C) before the equilibration experiment. Filter paper (Rotilabo®-round filters, type 11A, Germany), made of 100 % cellulose and bleached medical cotton (Paul Hartmann AG, Germany) were prewetted by spraying because the initially dry filter paper and cotton hardly adsorbed any humidity from the air. Both materials were then slightly oven dried for different times (ranging from 0 to 60 min) at 50 °C before the equilibration experiment to achieve a water content comparable to that of fresh silage and to create a water content gradient. According to the product information, the casein powder (My Supps GmbH, Germany) contained 90 % natural casein and a small amount of carbohydrates, while the commercial wheat flour contained 70.9 % carbohydrates, most of which was starch.

2.2 Unconfined water

Five isotopically distinct, unconfined waters were used. We term them very heavy, heavy, tap, light and very light waters according to their relative ranking of $\delta^{18/16}O$ and $\delta^{2/1}H$. These waters were produced from deionized water ($\delta^{18/16}O = -10‰$, $\delta^{2/1}H = -70‰$) by means of a rotary evaporator. Very heavy, heavy, light and very light waters had $\delta^{18/16}O$ values of 15, 2, −15 and −22 ‰, and $\delta^{2/1}H$ values of 125, 21, −113 and −160 ‰ with slight deviations between individual experiments.

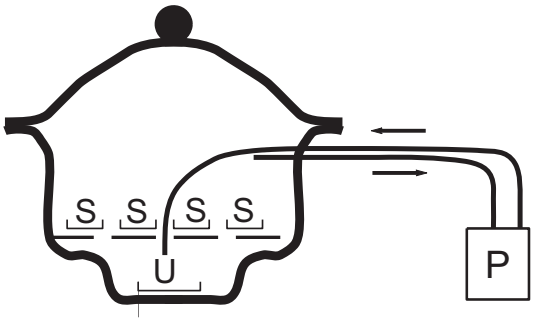

Figure 1. Experimental set-up with a desiccator vessel as the equilibration chamber. P: recycling pump ensuring air mixing and air movement within the chamber, U: unconfined water filled in the bottom part of the chamber, S: samples placed on top of the perforated middle plate. The arrows indicate the direction of air flow. Vaseline was used as sealant between the lid and the vessel.

2.3 Set-up of the equilibration procedure

The different materials were individually placed in closed chambers (glass desiccator vessels with a volume of approximate 20 L with drying agent removed) to equilibrate with unconfined water (Fig. 1). In a preliminary experiment, the effectiveness of the chambers' air seal was verified by flushing the containers with N_2, followed by monitoring the concentration of CO_2 and water vapour inside the vessels. The concentrations after closing the chamber remained constant, which indicated that leaks were negligible. In another preliminary experiment we assessed the development of humidity in the chamber. The humidity reached 100 % within 20 min (half-life 1.8 min) after we put 200 mL of water at the bottom of the chamber (Fig. 1), closed it and started the recycling pump (Laboport, Germany). All equilibration experiments lasted for 100 h. Sun et al. (2014) have shown that even for moist samples, equilibration is relatively fast (half-life 20 h). A preliminary experiment with silage showed no significant isotope difference ($p > 0.05$ for both H and O) in silage water between 60 and 100 h of equilibration, which implied that 100 h of equilibration were sufficient to achieve equilibrium conditions. Equilibrium conditions also imply that even if there had been condensation within the atmosphere-circulation system, it would not influence the isotope relation between dish water after equilibration and material water because the condensate would also be equilibrated.

In each experiment, 200 mL of (unconfined) water was placed in a glass bowl (15 cm in diameter) on the bottom of the chamber and dishes containing the material samples under focus (about 3 g fresh matter per dish) were placed on a perforated sill in the chamber. We flushed the chamber with nitrogen gas to remove the air vapour and the oxygen to prevent the decay of the samples. After that we immediately closed the chamber and started the recycling pump to ensure homogeneity within the airspace of the chamber. After 100 h of equilibration, samples were quickly removed from the chamber, placed in 12 mL glass vials sealed with a rubber stopper and wrapped with parafilm. The samples were then stored in a −18 °C freezer until water extraction by cryogenic vacuum distillation, as described by Sun et al. (2014). In addition, the weight of samples was recorded before and after extraction. During equilibration the unconfined water underwent changes due to the increase of humidity within the chamber (less than 0.3 % of the added water) and exchange with the varying amount of sample water (up to 10 %). To determine its isotopic composition when in equilibrium with the sample water, we sampled 1 mL of unconfined water at the end of equilibration and also subjected it to cryogenic vacuum distillation before measurement.

The extracted water was analysed by cavity ring-down (CRD) spectroscopy using a L2120 – i Analyzer (Picarro Inc., USA). Measurements were repeated until values became stable around a mean. Mean analytical uncertainties quantified as SD of different replicate measurements for each sample were ±0.06 ‰ for $\delta^{18/16}O$ and ±0.27 ‰ for $\delta^{2/1}H$. Post-processing correction was made by running the ChemCorrect™ v1.2.0 (Picarro Inc.) to exclude the influence of volatiles according to Martín-Gómez et al. (2015).

2.4 Experiment A: influence of materials

This experiment focused on the fractionation between water in different materials and unconfined water after equilibration. Dishes containing oven-dried silage, hay, oven-dried and fresh hemic litter, oven-dried and fresh fibric litter, filter paper, bleached medical cotton, casein powder or flour were all placed in different chambers for equilibration with unconfined water to avoid interference of volatiles in different materials. Eight samples for each material that differed in solid : water ratio were put in one chamber. Some materials (i.e. litter, filter paper, silage) were replicated in different experiments. The maximum number of samples for one material (silage) was 72. Flour and casein were powders and prone to form dust during vacuum water extraction. To prohibit this, the opening of vials containing flour and casein powder were covered by Parafilm with tiny holes.

2.5 Experiment B: influence of isotopic composition in unconfined water

This experiment aimed to find evidence that the isotopic fractionation was independent of the isotopic composition of the unconfined water. This independence will also prove that the isotopic fractionation cannot be caused by old water within the materials due to insufficient equilibration. Eight samples of oven-dried silage in each case were placed into chambers to equilibrate with five different unconfined waters.

2.6 Experiment C: pretreatment of silage

This experiment investigated the influence of volatiles on the isotope measurement and assessed the effect of silage solutes on isotopic fractionation between silage water and vapour. Fresh silage was divided into three groups (eight samples each): the first group did not undergo any pretreatment. For the second group, about 20 g of silage was immersed in 7 L of deionized water for about 2 min, stirred during immersion, then taken out using a colander and flushed with distilled water. After that we squeezed the silage by hand until no water drained off. This washing process was repeated three times. Finally, we reduced the water content of the washed silage by drying at 80 °C for 40 min. For the third group, silage was oven dried for 16 h at 100 °C to remove water and organic volatiles. These three groups (we call them fresh silage, washed silage and oven-dried silage hereafter) were placed in individual chambers and equilibrated with tap water for 100 h.

2.7 Statistics

For statistical evaluation we report two-sided 95 % limits of confidence (abbreviated CL) to separate treatments and ordinary least squares regression in order to describe relations between two variables. Measured values were fitted to expected relations by minimizing the root mean squared error (RMSE). Statistical requirements (normal distribution) were met in all cases. Significance, even if not explicitly stated, always refers to $p<0.05$.

2.8 Modelling

Conceptually, we assumed water to be part of one of two pools, which are arranged in a shell-like structure around the solid: an inner shell (or layer) which is in immediate contact or close to the surface of the solid and an outer layer that differs in thickness depending on the moisture content or solid : water ratio of the sample. Assuming that the outer layer has the same isotopic composition as the unconfined water once equilibrium was attained and that the inner layer has an isotopic composition that is influenced by the solid, the isotope composition of total adsorbed water (δ_T) was defined as follows:

$$\delta_T = f_O \times \delta_U + (1 - f_O) \times \delta_S, \quad (1)$$

where f_O is the fraction of water in the outer layer isotopically identical to the unconfined water, δ_U and δ_S are the isotope compositions of unconfined water and water influenced by the surface.

We defined isotopic fractionation ($\varepsilon_{S/U}$) between δ_S and δ_U.

$$\varepsilon_{S/U} = (\delta_S - \delta_U)/(1000 + \delta_U) \times 1000 \quad (2)$$

Combining Eqs. (1) and (2) leads to the following:

$$\delta_T = (1000 + \varepsilon_{S/U} \times f_O)/1000 \times \delta_U + \varepsilon_{S/U} \times f_O. \quad (3)$$

From this it follows that the apparent isotopic fractionation ($\varepsilon_{T/U}$) between the total water in the material and unconfined water is given:

$$\begin{aligned}\varepsilon_{T/U} &= (\delta_T - \delta_U)/(1000 + \delta_U) \times 1000 \\ &= (1 - f_O) \times \varepsilon_{S/U} = f_I \times \varepsilon_{S/U}. \end{aligned} \quad (4)$$

The fraction constituted by the inner layer f_I in Eq. (4) can be replaced by the ratio between R_I, the volumetric ratio of solid : water associated with the layer that is influenced by the surface, and R_T, the volumetric solid : water ratio of total adsorbed water:

$$\varepsilon_{T/U} = \varepsilon_{S/U} \times R_T/R_I. \quad (5)$$

Assuming that the size of the inner layer R_I, as well as $\varepsilon_{S/U}$, is constant for a certain material, $\varepsilon_{T/U}$ should be related linearly to R_T, which is the volumetric solid : water ratio for the total adsorbed water. The solid volume (exclusive voids) can be calculated by knowing the weight and particle density of the organic matters (casein: 1.43 g cm^{-3}, Paul and Raj, 1997; silage, hay, litter, filter paper, cotton and flour: 1.5 g cm^{-3}, Yoshida, et al., 2006).

In order to exclude that incomplete extraction had caused isotopic fractionation, we compared the observed isotopic fractionation with predictions based on a Rayleigh equation (Araguás-Araguás et al., 1995):

$$\varepsilon_{E/T} = (F^{1/\alpha} - F)/(F - 1) \quad (6)$$

where $\varepsilon_{E/T}$ is the predicted isotopic fractionation between the incompletely extracted water (E) and total water (T). F stands for fraction of water remaining in the material after the extraction and α stands for isotope fractionation factor (1.0059 and 1.0366 for $^{2/1}H$ and $^{18/16}O$ at 80 °C extraction temperature respectively).

2.9 Application case

Soil at 7 and 20 cm depths as well as rainwater were sampled at the grassland in Grünschwaige Experimental Station, Germany (48°23′ N, 11°50′ E, pasture #8 in Schnyder et al., 2006; 8.3 % organic matter, 30 % clay, 22 % sand) at biweekly intervals during the growing season (April to November) from 2006 to 2012 and at weekly intervals during the winter season (October to February) in 2015/2016. Soil sampling was always carried out on dry days at midday (between 11.00 a.m. and 16.00 p.m.). Two replicates of soil samples were collected on each sampling date. The data were used to examine (i) if there was an offset between soil water and rainwater and (ii) whether the offset can be corrected by accounting for the solid : water ratio according to our model. In order to exclude the possibility that the offset is caused

by soil evaporation, we only use winter season data. During the winter season, evaporation demand was low (average actual evaporation 0.5 mm d^{-1}, while average precipitation was 1.9 mm d^{-1}; German Weather Service, 2016) and evaporation demand should be entirely met by transpiration and intercepted water due to the complete grass cover. Growing season data are only shown for comparison. We had developed the relation between the volumetric solid : water ratio and the isotopic offset only for organic materials. These materials differed from the soil insofar as they did not contain minerals. Especially for sand, it can be expected that it practically does not absorb water due to its small surface area. Hence, we considered the sand to be inert and did not consider it in the volumetric solid : water ratio, which in consequence was calculated as follows: (volume of dry solid soil excluding sand) / (soil moisture volume). The volume of dry soil excluding sand was calculated by dividing its dry weight by particle density of the organic and mineral components (1.5 and 2.65 g cm^{-3} respectively; Blake, 2008).

3 Results

3.1 Experiment A: influence of materials

The apparent isotopic fractionation (sensu Eq. 4) of $\delta^{18/16}O$ and $\delta^{2/1}H$ was negative and significant ($p < 0.05$) for all materials, except for $^{18/16}O$ with filter paper and cotton and for $^{2/1}H$ in a few samples of cotton. The volumetric solid : water ratios differed between materials but also between different samples within the materials, providing a wide range. $\delta^{18/16}O$ and $\delta^{2/1}H$ apparent isotopic fractionation decreased significantly with volumetric solid : water ratio over the range of materials. The decrease was also significant for the different samples within each material (Fig. 2).

3.2 Experiment B: influence of isotopic composition in unconfined water

The isotope composition of absorbed water correlated closely with the unconfined water due to the wide range compared to the measurement errors ($R^2 = 0.9990$ and 0.9989 for $^{18/16}O$ and $^{2/1}H$ respectively; Table 1). However, the regressions showed that the intercept differed significantly ($p < 0.05$) from zero and the slope from one, which indicated that the isotope composition of adsorbed water was significantly different from that of unconfined water.

Equation (3) predicted a linear relation between δ_T and δ_U similar to the linear regressions shown in Table 1. In contrast to a regression, however, the slope and the intercept of Eq. (3) are not independent but depend on $\varepsilon_{S/U} \times f_O$. To account for this dependency, the slope and the intercept of the linear equations were estimated by adjusting $\varepsilon_{S/U} \times f_O$ in Eq. (3) to minimize RMSE, while fitting the measured δ_T and δ_U values. The optimal fits lead to the following:

$$\delta^{18/16}O_T = (1000 - 1.23)/1000 \times \delta^{18/16}O_U - 1.23$$
$$\delta^{2/1}H_T = (1000 - 22.6)/1000 \times \delta^{18/16}O_U - 22.6. \quad (7)$$

The R^2 between the predictions resulting from the two-layer model and the measurement were similar to that of the linear regression ($R^2 = 0.9990$ for $^{18/16}O$ and 0.9989 for $^{2/1}H$), although the model has one degree of freedom less than the regression. The resulting optimal $\varepsilon_{S/U} \times f_O$ values were -1.23 ‰ for ^{18}O and -22.6 ‰ for ^{2}H meaning that the effect was 18 times stronger for ^{2}H than for ^{18}O.

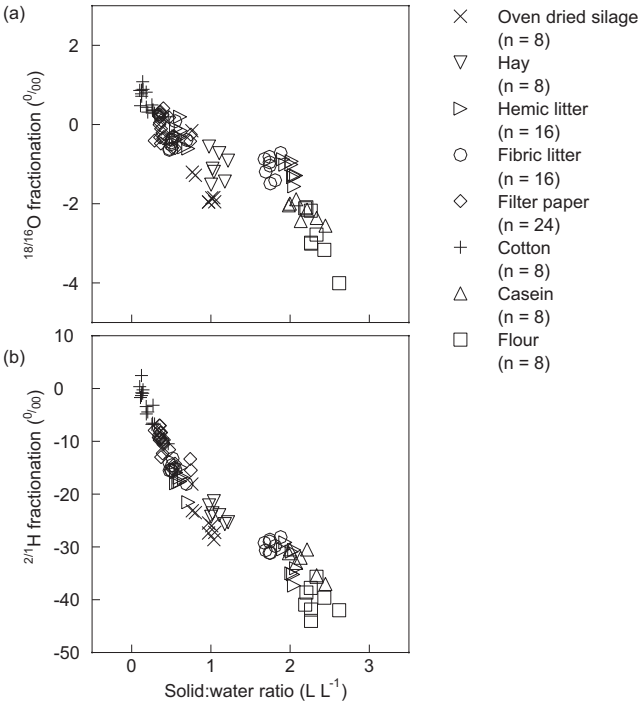

Figure 2. Relationship between volumetric solid : water ratio and apparent isotopic fractionation of **(a)** $^{18/16}O$ and **(b)** $^{2/1}H$ between unconfined water and total water adsorbed by different materials. Taken together, the regressions are $y = -0.906\,x$ ($R^2 = 0.6789$; $N = 96$) for the isotopic fractionation of $^{18/16}O$ and $y = -17.75\,x$ ($R^2 = 0.8355$) for the isotopic fractionation of $^{2/1}H$.

Table 1. Regressions between water adsorbed by silage (δ_T) and unconfined water (δ_U) for five types of water (very heavy, heavy, tap, light and very light water) based on equation $\delta_T = \text{slope} \times \delta_U + \text{intercept}$; $n = 40$; values in parenthesis denote the 95 % confidence level.

	$\delta^{18/16}O$	$\delta^{2/1}H$
Intercept	−1.30 (±0.14)	−22.9 (±1.1)
Slope	0.987 (±0.010)	0.968 (±0.011)
R^2	0.9990	0.9989

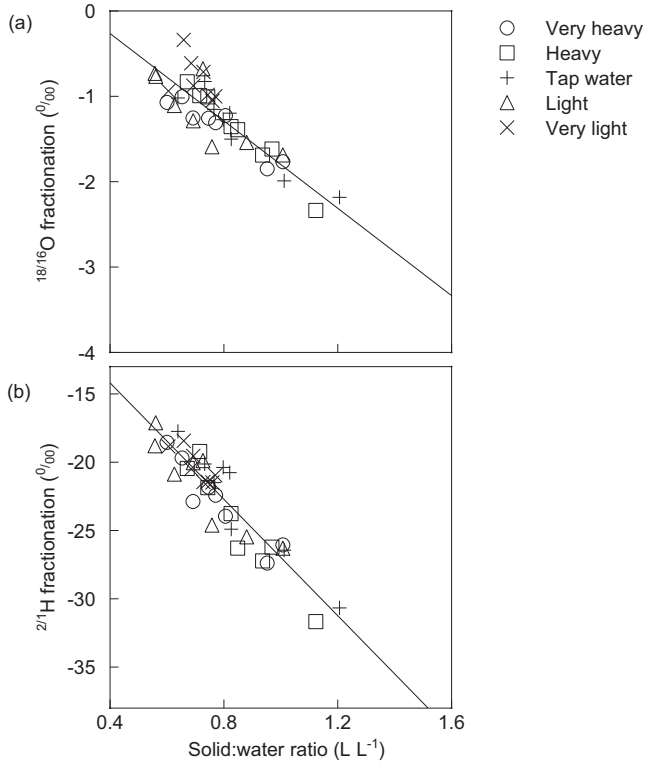

Figure 3. Relationship between volumetric solid : water ratio and apparent **(a)** $^{18/16}$O and **(b)** $^{2/1}$H isotopic fractionation of total water absorbed by silage compared to unconfined waters with different isotopic composition. The lines show the best fit (see Eq. 7).

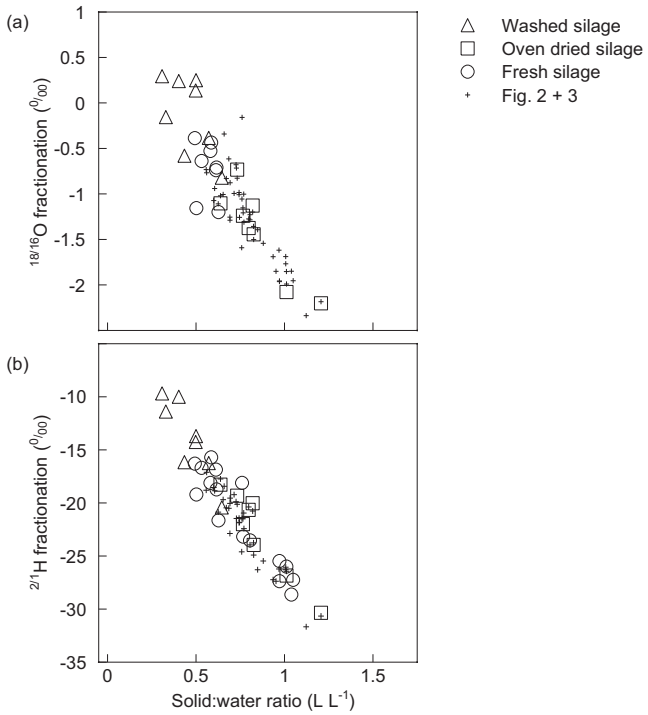

Figure 4. Relationship between volumetric solid : water ratio and the apparent isotopic fractionation of **(a)** $^{18/16}$O and **(b)** $^{2/1}$H between unconfined water and total water adsorbed by silage with different pretreatments ($N = 8$ each). The data of Figs. 2 and 3 (both oven-dried silage, $N = 32$) are provided for comparison.

Equation (5) predicted that the apparent isotopic fractionation changes linearly with the solid : water ratio. This relation was highly significant ($p < 0.01$) also in the case when waters with very differently isotopic composition were used (R^2: 0.7589 and 0.8599 for $^{18/16}$O and $^{2/1}$H respectively; Fig. 3). These relations were identical for very heavy, heavy, tap, light and very light water.

3.3 Experiment C: pretreatment of silage

There was no significant difference between mean gravimetric water contents (based on dry matter) of washed silage (153 % ± 33 %) and fresh silage (128 % ± 10 %) after 100 h equilibration. The water content of oven-dried silage did not remoisten the same water content as fresh silage but remained significantly drier (81 % ± 13 %). The apparent isotopic fractionation of washed silage, oven-dried silage and fresh silage all decreased with the solid : water ratio (Fig. 4), as already noted in the experiment with different materials (Fig. 2) or in investigations with unconfined waters of different isotopic composition (Fig. 3). Washing and oven-drying should have removed most solutes and volatiles and thus have created a large variation in the amount of solutes and volatiles among the treatments. Still, the relationship between apparent isotopic fractionation of all three types of silage and solid : water ratio followed the same line and the areas overlapped each other for the three types of silage (Fig. 4). This implied that neither the volatiles, which possibly could have adulterated the measurements, nor the solutes, which possibly could have influenced water activity in the silage, were the cause of isotopic fractionation. The different treatments, however, separated along the common line due to their differences in water content, which again corroborated the prediction that the apparent isotopic fractionation should linearly change with solid : water ratio.

3.4 Combining experiments A, B and C

When combining all experiments with different materials, different pretreatments and different unconfined waters, apparent isotopic fractionation covered a wide range of about 5 ‰ for $^{18/16}$O and 46 ‰ for $^{2/1}$H (Fig. 5). Even within the same materials, the range was up to 2.5 ‰ for $^{18/16}$O and 25 ‰ for $^{2/1}$H. Apparent isotopic fractionation within materials linearly decreased with the volumetric solid : water ratio.

The isotopic fractionations predicted for Rayleigh fractionation were far from the observed isotopic fractionations (Fig. 5). The average deviation between the expected and the

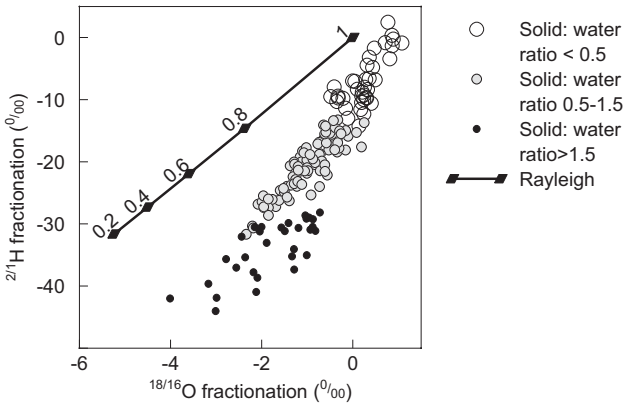

Figure 5. Apparent isotopic fractionation ($^{2/1}$H vs. $^{18/16}$O) of extracted water as observed in all experiments (markers indicate three groups of solid : water ratios) and fractionation as expected from Rayleigh fractionation (line; numbers denote the fraction of extracted water).

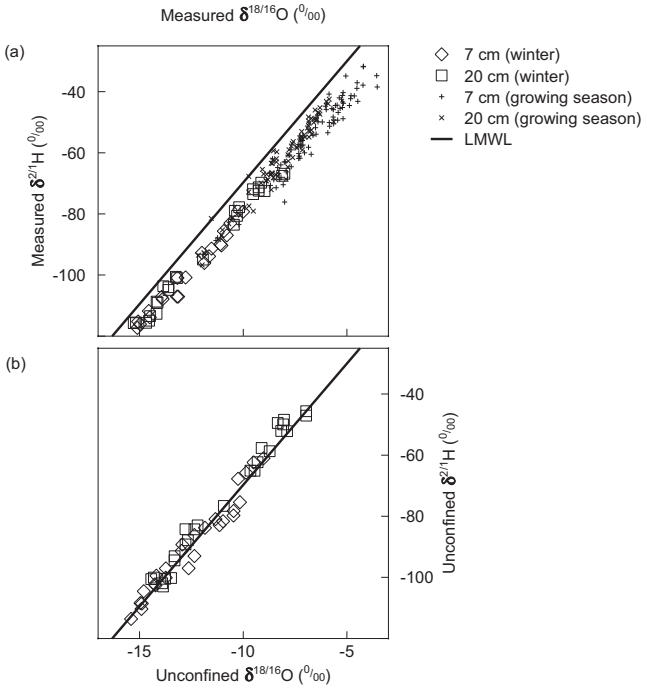

Figure 6. Isotope composition of soil water at 7 and 20 cm depth (winter season: $N = 26$; growing season: $N = 48$). **(a)** Measured total soil water. **(b)** Estimated unconfined water. The solid line denotes the local meteoric water line ($N = 79$; $y = (8.0 \pm 0.2)\,x + (10 \pm 2)$; $R^2 = 0.99$).

observed $^{2/1}$H isotopic fractionation was about 15 ‰. Furthermore, the slope of the relation between the fractionation of $^{2/1}$H and $^{18/16}$O was significantly steeper ($p < 0.05$) for the observed enrichment than the slope predicted for a Rayleigh process. Additionally, the average $^{2/1}$H fractionation of the materials was −20.6 ‰. This net fractionation could be expected for a Rayleigh process if only 80 % of the water had been extracted while 20 % remained in the sample. This, however, was not the case because subsequent oven-drying did not cause further weight loss.

3.5 Application

For the growing season, soil water at 20 cm depth and 7 cm depth showed a distinct deviation from the local meteoric water line (mean deviation for $^{2/1}$H : −8.1 ‰) with a slope almost identical to that of the meteoric water line (Fig. 6a). An identical mismatch was detected for the winter season (markers in Fig. 6a), for which confounding effects of evaporation are minimal, and for the summer season.

The deviation between the winter season data and the local meteoric water line correlated significantly ($p < 0.001$) with the solid : water ratio for 7 cm depth but not for 20 cm depth, which varied less in water content. For both depths, the data moved closer to the local meteoric water line when the influence of confined water was removed by applying the general regression with solid : water ratio from Fig. 2 (Fig. 6b). The mean deviation for $^{2/1}$H changed from −8.1 to 1.0 ‰ for both depths due to this correction.

4 Discussion

The extraction of water from solid–water mixtures can be biased by incomplete extraction (Araguás-Araguás et al., 1995) or by the exchange of hydrogen or oxygen from the soil material with water molecules (Meißner et al., 2014). Here we add another confounding effect, which is the inhomogeneous isotopic composition of water above a solid surface. In the following we will discuss (1) whether the observed effect can be due to measuring errors or reasons other than the proposed surface effect, (2) possible reasons for the surface effect, (3) the fields of application for which this surface effect will likely be important and (4) further work related to the surface effect which may follow.

4.1 Excluding mechanisms other than the proposed surface effect

The study provided clear evidence that the water adsorbed by organic surfaces differed from what would be expected from the isotopic composition of unconfined water and it showed that this deviation became larger with decreasing water content. Alternative mechanisms leading to an isotopic fractionation other than the proposed surface effect could be (a) volatiles adulterating the measurements, (b) solutes influencing the isotopic composition of adsorbed water, (c) insufficient equilibration time, (d) incomplete extraction of water, (e) metabolically produced water from microorganisms adhering to the materials, (f) exchange of hydrogen and oxygen between the organic matter and the adsorbed water.

(a) The surface effect was largest for flour and casein, which do not produce volatiles. The filter paper and cotton, which contain no volatiles, also showed a decreasing trend between apparent isotopic fractionation and solid : water ratio (Fig. 2). Even for silage the influence of volatiles was not evident because washed or oven-dried silage, which should have lost all its volatiles, behaved identically to fresh silage. Also the error in water content caused by not accounting for volatile losses was negligible. The correction function by Porter and Murray (2001) to calculate the true water content from the loss of weight moves the respective data points of silage in Fig. 4 only invisibly (about $0.03\,L\,L^{-1}$ towards the right side).

(b) Solutes in water can influence the isotopic fractionation between water and vapour because the energy stage of water molecules bound in the primary hydration sphere of cations and anions differs from that of the remaining bulk water molecules (Kakiuchi, 2007). This effect has been shown for many salts (e.g. KCl, NaCl, Na_2SO_4 and $ZnSO_4$). The strength of this effect varies between different ions and may be small (Kakiuchi, 2007; Sofer and Gat, 1975; Stewart and Friedman, 1975). NaCl even does not have a measurable effect on $^{18/16}O$ (O'Neil and Truesdel, 1991). Most of the solutes in our materials were organics for which the effect is unknown. However, this effect must have been small as the washed silage did not show a different pattern in isotopic fractionation compared to fresh silage (Fig. 4). Also the filter paper of analytical grade and the bleached cotton, both of which should not carry any solutes, did not show a different pattern.

(c) Insufficient time for equilibration may especially be relevant for silage and litter, which had the highest initial water content. For silage we could show that the apparent isotopic fractionation was independent of the isotopic composition in the unconfined water (Experiment B) despite the wide range of differently labelled unconfined waters (range for $^{18/16}O$: 32 ‰; range for $^{2/1}H$: 285 ‰). However, any old water would have led to a separation in the apparent isotopic fractionation. In contrast, our results were in accordance with the general rule that isotopic fractionation is independent of the isotope composition of the source, which also underlies Eqs. (4) and (5). Furthermore, all our experiments used deionized water prepared from tap water, except for the experiment with labelled waters for which we can exclude the existence of old water. Our deionized water was similar in isotopic composition to silage water and soil water. The mean $\delta^{18/16}O$ of our water was −10 ‰ while the mean for 52 fresh silage samples analysed by Sun et al. (2014) was −11 ‰ (SD 3 ‰). A small fraction of old water thus cannot cause the large observed effects.

(d) An incomplete extraction should cause a large error at low moisture content, similar to the general relation between solid : water ratio and isotopic fractionation that we have observed (Fig. 5). However, the predicted isotopic fractionation by incomplete extraction based on a Rayleigh fractionation deviated from the observed isotopic fractionation (Fig. 5). In addition, no significant weight difference before and after oven-drying of the samples was observed after vacuum extraction. Incomplete extraction is thus an unlikely explanation.

(e) Kreuzer-Martin et al. (2005) found that 10 % of the total water extracted from *Escherichia coli* cells during the log-phase of growth was generated by metabolism from atmospheric oxygen. Thus, intracellular water was distinguishable from extracellular water in $\delta^{18/16}O$. We flushed the chambers with nitrogen gas before equilibration to reduce availability of atmospheric oxygen and minimize microbial growth. For materials like silage dried at 100 °C or filter paper, any significant microbial growth is unlikely. Furthermore, isotopic adulteration caused by microorganisms should have caused $^{18/16}O$ and $^{2/1}H$ deviations in the opposite direction for the very heavy and the very light labelled experiments akin to the experiments by Kreuzer-Martin et al. (2005). In contrast to this, ^{18}O and ^{2}H were always depleted in our experiments regardless of the isotope composition of unconfined water.

(f) Hydrogen bound to oxygen and nitrogen in many organic materials like bitumen, cellulose, chitin, collagen, keratin or wood may exchange isotopically with ambient water hydrogen (Bowen et al., 2005; Schimmelmann, 1991). At room temperature, this isotopic exchange occurs rapidly in water and an exchange with vapour is even several orders of magnitude faster (Bowen et al., 2005; Schimmelmann et al., 1993). Such an exchange would influence the adsorbed water but it would also influence the unconfined water, which is in equilibrium with the adsorbed water but could not influence the fractionation between them. The same would apply for an exchange between carbonate oxygen and water oxygen (Savin and Hsieh, 1998; Zeebe, 2009), although our samples did not contain any carbonate.

4.2 Possible reason for the surface effect

The isotopic fractionations became more negative with increasing solid : water ratio and followed the predictions of Eq. 5. This implied that similar isotopic fractionations existed in different materials and that the simple two-layer model sufficiently described the experimental values. Abundant evidence exists that the properties of water change close to a surface (Anderson and Low, 1957; Goldsmith and Muir, 1960; Miranda et al., 1998). A hydrogen-bonded ice-like network of water grows up as the relative humidity increases.

Above 60 % relative humidity, the liquid water configuration grows on top of the ice-like layer (Asay and Kim, 2005). This transition from two-dimensional ice-like water to a three-dimensional water-like layer has been already been shown in several cases (Kendall and Martin, 2005). As we used 100 % relative humidity in our chamber, both layers should have been present.

The anomalies of water close to a surface appear not to be particularly affected by the detailed chemical nature of the solid substrates with which the water is in contact. This is referred to as the "paradoxical effect", which describes how – independent of the nature of the surface – water close to a solid interface is characterized by long-range ordering including high-pressure ice polymorphs of low energy (Drost-Hansen, 1978). This agrees with our observation that the difference between materials was small compared to the large variation of the effect caused by a varying solid : water ratio. The small differences between materials that appear in Fig. 2 may hence only be an effect due to differences between the different materials in their specific surface area per volume of solid but not due to their chemical nature. The water content of oven-dried silage (81 % ± 13 %) did not reach the same water content as fresh silage (128 % ± 10 %) but remained significantly drier, which may be because oven-drying changes the surface roughness and other structural properties of silage (Tabibi and Hollenbeck, 1984).

In accordance with our study, Richard et al. (2007) found that water adsorbed in porous silica tubes was depleted in ^{2}H compared to unconfined water and depletion increased with decreasing water quantity as a result of the interplay of molecular vibrational frequencies and intermolecular H-bonding. This mostly depends on the difference in zero-point energy between the $^{16/18}O$–$^{1/2}H$ bonds, which is compressed at the transition between the bulk liquid and the confined liquid influenced by the surface (Richard et al., 2007). Our data show, that the effect is much larger for $^{2/1}H$ than for $^{18/16}O$ and it practically disappears for $^{18/16}O$ when the solid : water ratio decreases below 0.5 (Fig. 5). This may explain why the effect has been previously described for $^{2/1}H$ but not for $^{18/16}O$. Oerter et al. (2014) investigated water adsorbed to clay and also found isotopic fractionation. They explained this by the negatively charged clay surface, which increases the ionic strength in the solution close to the clay surface. Ions are known to cause fraction in their hydration sphere (Kakiuchi, 2007; Stewart and Friedman, 1975). This mechanism could also be active in our samples, although the surface charge of most of our samples (e.g. cellulose) is much smaller than surface charge of clays. Washing, which should have removed most of the solutes, did not remove the fractionation.

4.3 Fields of application

In our experiments we have only examined organic materials while the soil in our application case also contained minerals. Given the "paradoxical effect" (Drost-Hansen, 1978) and that we had not found any effect of the nature of the organic materials on the surface effect, the simplest assumption was that there is also no large difference between organic and mineral surfaces regarding the isotope effect. This seemed reasonable because pure clay with 30 % water content (equivalent to 0.8 solid : water content) as used by Oerter et al. (2014) created −0.4 ‰ oxygen isotopic fractionation on average. This was close to the predicted apparent isotopic fractionation (−0.7 ‰) for the same solid : water ratio for organic materials. Oerter et al. (2014), however, also manipulated the composition of the solutes, which are known to affect fractionation and do not allow direct comparison.

The isotopic composition of water in porous samples is usually determined by extracting all water in order to avoid any shift caused by Rayleigh fractionation. Hence, the inner layer close to the surface and the outer layer will be mixed. We could not estimate the thickness of inner layer for our experimental materials. The high-pressure ice polymorphs near surfaces may be one tenth of a micrometer in thickness (Drost-Hansen, 1978) but other effects at the surface–water interface, like effects on solute composition, extend to a scale of tens of micrometers and in extreme cases up to 0.25 mm (Zheng and Pollack, 2003).

For many processes, especially in the transport of liquid water (e.g. groundwater recharge, stream flow discharge, water uptake by plants), only the outer, mobile layer will be relevant. The extraction of total water will then give a biased estimate of the mobile water. In accordance with our hypothesis, Brooks et al. (2010) even suggested two different soil water worlds to explain their data (mobile water and tightly bound water), which were not identical in terms of isotope composition. They also measured soil water collected in low-tension lysimeters, which represents mobile water, and bulk soil water extracted cryogenically. Bulk soil water was always more depleted in heavy isotopes than lysimeter water collected at the same depth, which was in line with the isotopic fractionation direction observed in our soil case. Tang and Feng (2001) also found isotopic differences between mobile and immobile water in soil and explained this by incomplete replacement of soil water by rainwater. Our laboratory experiments aimed to exclude such an effect. In our application case we also found a consistent offset between rainwater and soil water that cannot result from incomplete replacement of old rainwater in soil with new rainwater because soil water had an offset from the meteoric water line. Such an offset has been shown for many locations around the world (Brooks et al., 2010; Evaristo et al., 2015), which challenges the assumption in land surface models that plants and streams derive their water from a single, well-mixed subsurface water reservoir. Additionally, the surface effect may also play a role in the fractionation between source water and xylem water that has been described for some xerophytic and halophytic species (e.g. Ellsworth and Williams, 2007) for which an explanation is presently missing.

In other cases, which focus on the liquid–solid interface, only the water of the inner layer, which is influenced by the surface effect, will be relevant. For example, in studies of cell wall formation or degradation, the total water should be a biased estimate of the isotopic composition near the cell wall. Due to the change in apparent isotopic fractionation with water content, the total cell water will change just by a variation in vacuole volume even if the isotopic composition near the cell wall and in the vacuole remain unchanged. Another example is the determination of exchangeable hydrogen in organic tissues, which is needed to trace the origin of animals (such as the protein in hair, Bowen et al., 2005). This is usually determined by exposing the tissue to vapour in equilibrium with either heavy or light water similar to our experiments. The surface effect may thus also play a role for the exchangeable hydrogen.

4.4 Further work

Solid : water ratio is clearly not the best parameter to describe the two-layer model. The relation should be influenced by specific surface area and wettability. Hence, the water volume per wetted surface area would likely be a better parameter. For instance, when we wet the filter paper inhomogeneously, we obtained random results because the average solid : water ratio neither reflected the situation of the wet spots nor that of the dry spots. Furthermore, the increasing scatter for solid : water ratios > 1.5 (Fig. 5) likely resulted from an inhomogeneous water distribution in these rather dry samples that may have left some parts of the sample completely dry and thus underestimated the water content of other parts. Still, our model was easy to apply and it worked sufficiently for the wide variety of materials examined. More materials varying in hygroscopic/hydrophobic behaviour and in surface area should be included to better understand the rule behind the variation of isotopic fractionation and to expand the model.

5 Conclusions

There was an abundance of evidence to suggest that the surface effect influenced the isotopic fractionation between water adsorbed by organic matter and unconfined water. Many hypothetical reasons for an erroneous isotopic fractionation could be excluded. The variation of apparent isotopic fractionation with water content was well described by a simple, easy to apply two-layer model. This isotopic fractionation should not be neglected when the surface area is huge and the water content is low. The surface effect will become especially relevant for processes happening at the liquid–surface interface like the growth or degradation of the organic materials.

Author contributions. Guo Chen and Karl Auerswald designed the experiments and analysed the data. Guo Chen carried out the experiments and wrote a first draft. All authors developed and approved the manuscript.

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Article

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Ambient Conditions and Feeding Strategy Influence $\delta^{18}O$ of Milk Water in Cows (*Bos taurus*)

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Supporting Information

ABSTRACT: There are increasing concerns by consumers regarding agricultural product traceability and authenticity. Oxygen isotope composition ($\delta^{18}O$) has been used in this context based on the relationship between $\delta^{18}O$ of animal products and annual precipitation. However, in dairy products this relationship is affected by the seasonality of $\delta^{18}O$ in milk water which in turn depends on the feeding system used. We measured 608 milk samples from 28 farms with various feeding strategies in southern Germany throughout the year, investigating the influences of ambient conditions, drinking water source, and feeding strategies on seasonal variation of $\delta^{18}O$ in milk water (δ_{milk}). The mechanistic Munich–Kohn model reflecting these influences predicted the seasonal and farm-specific variation of δ_{milk} well. The relationship between $\delta^{18}O$ of precipitation and δ_{milk} varied in different feeding strategies. The interplay of ambient conditions and feeding strategy on δ_{milk} should thus be carefully considered when identifying the origin of milk.

KEYWORDS: *proficiency, animal environment, animal keeping, silage, grass*

INTRODUCTION

The frequent and global exchange of food provides consumers with a wide range of choices, but this exchange may also present some risks. These include the potential for rapid spread of food-borne diseases and opportunities for sales of contaminated food, including, for example, residues of pesticides that may be banned in one country but permitted in another. Thus, there is a growing demand among consumers for safe and high-quality food, including products that have a clear regional identity and production strategy. This requires reliable authentication tools. The variation in the stable isotopic composition in food has been suggested as useful for testing the authenticity of many food products, and it has been used for cheese, fruit, juice, oil, and meat.[1−6]

Among human foodstuffs, milk and milk products are in high demand and may attain premium prices, but they originate from innumerable farmers, and this may increase the risk of adulteration.[7] A significant linear regression between the isotope composition of oxygen $\delta^{18}O$ (or $\delta^{2}H$) of milk water and tap water, as found in the U.S.A., may be used to trace the origin of products because the $\delta^{18}O$ of tap water reflects the spatial variation found in precipitation.[8] However, δ_{milk} exhibits a seasonal variation. For instance, it was found to range from −8 ‰ to −4 ‰ on farms in southern Germany.[9] Such seasonal variation is superimposed on the spatial variation, thereby increasing the difficulty of identifying the origin of products.[10] Furthermore, the conditions under which cattle are fed and kept may also modify this variation. Understanding the reasons and predicting the degree of seasonal variation in δ_{milk} are thus the prerequisites for testing the authenticity of milk.

Milk water originates from body water of lactating cows, and thus it reflects the influences of different water sources available to the cow and also the different pathways of water losses by the cow. Abeni et al.[11] reported that δ_{milk} is higher in summer than in winter under the same feeding strategy, and they attributed this to the ^{18}O enrichment by the greater losses of transcutaneous water and respiratory water exhaled in breath in summer. Kornexl et al.[9] and Magdas et al.[12] suggested that the seasonal variation originated from the more enriched water in plants in summer than in winter. Thus, the feeding strategy also influences the $\delta^{18}O$ of the O input flux. In temperate zones with distinct seasons, fresh grass, either grazed or cut, is available in summer while silage is often the main feed for cows in winter. On some farms, silage may also be an important source of feed in summer. Water from fresh grass and silage differs considerably in isotope composition.[13] Thus, Renou et al.[14] found a significant difference in δ_{milk} for different feeding diets (fresh grass and silage), while in another case δ_{milk} was not significantly influenced by the dietary composition, probably being more related to the geo-climate.[15] It appears that the seasonal variation of δ_{milk} is attributed to both ambient conditions and feeding strategy. The relative importance of these two causes may differ in different circumstances because of three spheres of influence: (1) The amounts of drinking water and feed moisture change with season and feeding strategies, which can be grouped into three typical feeding strategies: grazing on pasture, feeding on cut grass in stalls, or feeding on silage in stalls. To date, no studies have explicitly analyzed the impact of these feeding strategies on δ_{milk}. (2) $\delta^{18}O$ of feed moisture varies regionally, seasonally, and diurnally. For example, the enrichment of ^{18}O in leaf water by plant transpiration varies depending on the extent of plant transpiration[16] but even $\delta^{18}O$ in silage water varies due to an exchange with air humidity.[13] (3) The animal response to environmental and production conditions influences both its

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water uptake and its water losses, with respective influences on body water $\delta^{18}O$. For example, drinking water uptake decreases if there is rainwater or dew adhering to the ingested grass;[17] transcutaneous water losses increase water demand[18] but also enrich body water in heavy O.[19] The multitude of influences makes it necessary to use a model that accounts for the mutual interactions of ambient conditions and the different feeding and keeping strategies on the variation of δ_{milk}.

A mechanistic model (the Munich−Kohn model or MK model), extended from the Kohn model[19] that was based on the isotopic balance and amount balance of O, has already been established and applied to the hair of a suckler cow kept on a farm under temperate climate conditions (Grünschwaige Experimental Station; 48°23′N, 11°50′E) in our previous work.[20] An advantage of this model is that it considers more species-specific parameters than the general Kohn model (e.g., milk production, keeping conditions, and the turnover of body water). However, previously it was tested in the hair of only one suckler cow with two feeding strategies[21] (grass grazing and grass silage feeding), and it requires further testing. This calls for the inclusion of other feeding strategies, especially feeding of maize silage, which is a major component of many cattle diets but for which no data on $\delta^{18}O$ in silage water are presently available. Further, testing a mechanistic model requires the inclusion of situations that deviate from typical collinearity of some input parameters. For instance, precipitation usually influences feedwater, tap water, and air humidity simultaneously. This restricts the disentangling of the individual contributions of these water sources to $\delta^{18}O$ in body water. Situations where tap water is not derived from local precipitation but from lake water or paleo-water provide opportunities for validating the MK model regarding input parameters that are often but not necessarily correlated. In summary, further tests of the MK model using different husbandry conditions, feed components, and sources of drinking water are thus necessary for its wider validation but also for a better understanding of the influences on δ_{milk}.

In this study, $\delta^{18}O$ of milk water from 28 farms in southern Germany, with different sources of drinking water and large seasonal and farm-to-farm contrasts in feeding strategies, was investigated. The relationship between δ_{milk} and ambient conditions under different feeding strategies was compared, and the feasibility of the MK model was tested.

MATERIALS AND METHODS

Site Description. We investigated 28 farms in southern Germany covering an area of about 80 km N−S and 370 km E−W (Figure 1) with altitudes ranging from 384 to 698 m. The farms were separated into two groups according to altitude (high/low, divided at 600 m).

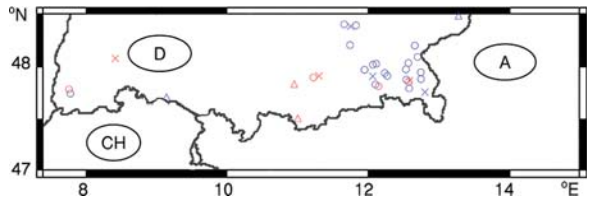

Figure 1. Locations of farms (circles), meteorological stations (crosses), and isotope stations (triangles). D, CH, and A denote Germany, Switzerland, and Austria, respectively. Red and blue markers represent high (>600 m) and low (<600 m) altitude, respectively.

Daily weather data were obtained from six nearby stations operated by the German Weather Service, at locations shown in Figure 1, which also were separated according to high and low altitude. Temperature (annual range between monthly averages: −3 to +19 °C), vapor pressure deficit (range: 50−694 Pa), and relative humidity (range: 66−90%) exhibited little variation between stations with slightly lower temperatures (on average 0.7 °C) at higher altitude.

Data on $\delta^{18}O$ in precipitation were taken from two stations at a high altitude (Hohenpeissenberg 977 m a.s.l.; Garmisch-Partenkirchen, 719 m a.s.l.) and two stations at a low altitude (Konstanz, 443 m a.s.l.; Passau-Fuerstenzell, 476 m a.s.l.; locations are in Figure 1).[22] High altitude means were 1‰ lower than low altitude means ($P > 0.05$; annual range between monthly averages: −16 to −5‰) while there were no significant differences within each group.

Feed Components and Feeding Strategies of Farms. Feed composition (grass silage, hay, maize silage, concentrates, and fresh grass either from pasture or cut) was obtained from interviews with the farmers. On 13 farms, both feed transitions from conserved winter feed (silages and hay) to summer feed (including fresh grass, either cut or from pasture) and back to winter feed were recorded. On the other 10 farms only the transition in spring from winter feed to summer feed was captured. We defined the time when fresh grass was afforded as warm seasons and the time when no fresh grass was afforded as cold seasons on the above 23 farms. On the remaining five farms, constant feeding of silage, hay, and concentrates was used, and May to September was defined as warm season in these cases.

For simplicity, we grouped the strategies into three classes depending on the main feed component: grass fed on pasture, stall feeding of cut grass, and feeding no fresh grass (only silage or hay was fed in a stall; this included all farms during winter feeding and five farms during summer feeding). For brevity, we refer to these as strategies of pasture grass, cut grass, and no grass, respectively. Each farm practiced one to three feeding strategies within a year. More information about the farms, the feed, and the milk was reported previously.[23]

With few exceptions the farms used tap water taken from local groundwater to supply water for the cows. Farms 6, 27, and 28 received their tap water from waterworks that extracted their water near the shoreline downstream from a large lake. Waterworks supplying farm 12 used paleo-water that was about 5000 years old and extracted from 100 to 120 m depth from the second and third groundwater storey. During the grazing season, farms 10 and 11 located in a mountain area used spring water to provide animals with drinking water on their pastures; this water resulted from subsurface flow on bedrock underneath a shallow soil with short flow lengths (maximum distance to crest <500 m), while in stall seasons they used tap water.

Sampling of Milk. Well-mixed tank milk was sampled weekly on these 28 farms in 2005. On 15 farms sampling covered the period from March 29th to December 13th while on the other 13 farms, sampling covered only the period before May 17th (Figure 2). The milk yield was recorded per cow (7012 samples). Energy corrected milk was obtained by adjusting to 3.5% fat and 3.2% protein. The experiment involved no interaction with the animals and thus there were no ethical approval requirements.

Milk samples were immediately frozen and stored at −20 °C. For $\delta^{18}O$ analyses, milk samples were thawed and homogenized by vortexing with 8000 rpm to prevent buildup of a fat layer. Aliquots (200 μL) were pipetted into 3.7 mL vials, and 2 mg of benzoic acid was added to prevent coagulation.[9] The benzoic acid had an ignorable influence on the measurement because the ratio of milk O to (exchangeable) benzoic-O is 10000:16. The $^{18}O/^{16}O$ ratio in milk water was measured by an IsoPrime isotope ratio mass spectrometer (GVI, Manchester, U.K.) that was interfaced to a multiflow equilibration unit (GVI, Manchester, U.K.). O isotope data are presented as $\delta^{18}O$ (‰), where $\delta^{18}O = (R_{sample}/R_{standard} - 1)$, with R the $^{18}O/^{16}O$ ratio in the sample and in the standard (V-SMOW; Vienna Standard Mean Ocean Water), respectively. After every 10 samples, two laboratory water standards ($\delta^{18}O$ +15.5‰ and −20.5‰), previously calibrated against VSMOW, VGISP, and

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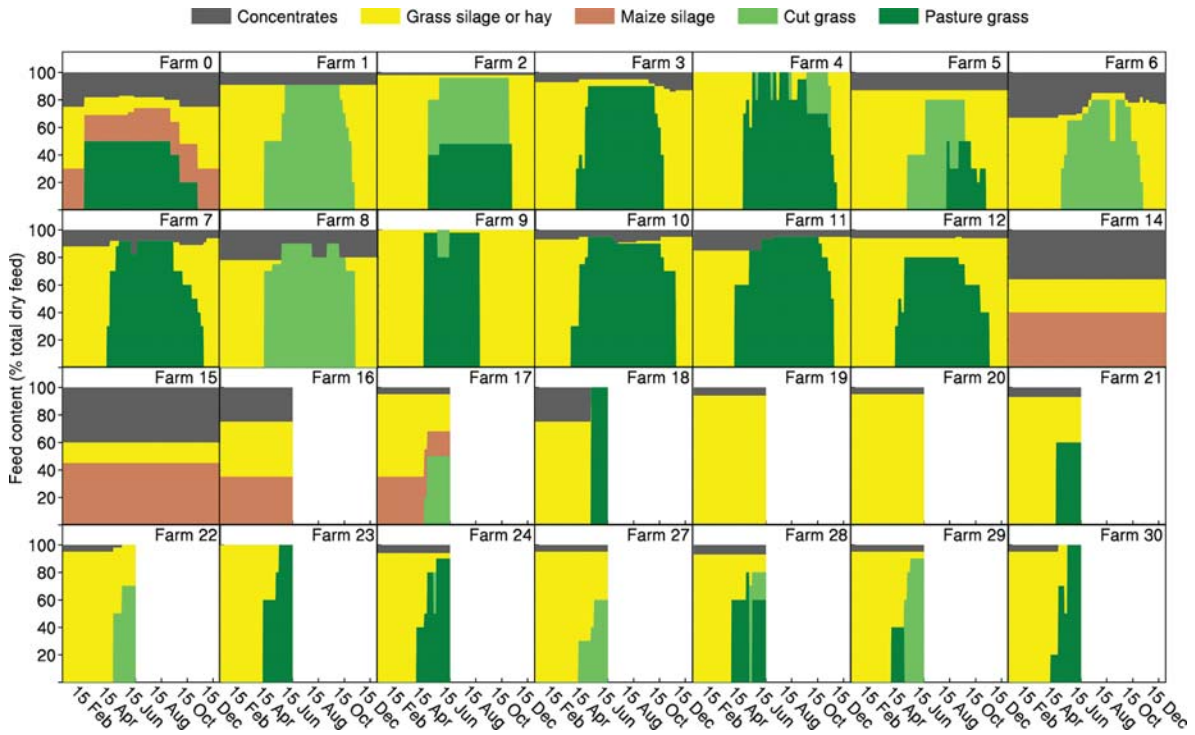

Figure 2. Seasonal variation of feed composition in different farms.

VSLAP, were run for possible drift correction and SMOW scaling. Standard deviation for the measurement of laboratory water standards during the measurement period was 0.16‰.

We sampled tap water (N = 22) and additionally spring water from farms 10 and 11. Additionally, maize silage (N = 18), grass silage (N = 42), and hay (N = 9) within our experimental regions were sampled, and we extracted the water with custom-made cryogenic vacuum distillation as described in Liu et al.[24] The drinking water and extracted water from silage and hay were analyzed with a Cavity Ring Down Spectrometer using a L2120i Analyzer (Picarro Inc., Santa Clara, CA). Measurements were repeated (more than four injections) until values became stable, and the last two measurements were averaged (average SD of different replicate measurements for each sample was ±0.06‰). After every 20−25 samples, two laboratory water standards were measured for possible drift correction and normalizing results to the VSMOW scale. Postprocessing correction was made by running the ChemCorrect v1.2.0 (Picarro Inc., Santa Clara, CA) to exclude the influence of volatiles.[25]

Modeling. According to the MK model[20] the $\delta^{18}O$ of the body water in a cow (δ_{bw}) on any day i is given by the body water composition of the previous day ($i - 1$) and the input and output fluxes of O isotopes of day i given by their molar masses M and their isotopic composition.[20]

$$(M_{\text{input}O}\delta_{\text{input}O} + M_{\text{bw},i-1}\delta_{\text{bw},i-1})/(M_{\text{input}O} + M_{\text{bw},i-1}) = (M_{\text{output}O}\delta_{\text{output}O} + M_{\text{bw},i}\delta_{\text{bw},i})/(M_{\text{output}O} + M_{\text{bw},i}) \quad (1)$$

Input fluxes comprise air O uptake, air water vapor intake into the lungs, intake of chemically bound O with digested feed, and intake of feed moisture and of drinking water. Output fluxes include CO_2 production, fecal water, organic products, respiratory water, milk water, sweat, transcutaneous water vapor, urea, and urinary water. The $\delta^{18}O$ of fecal water, milk water, sweat water, and urinary water can be simply replaced by $\delta_{bw,i}$ because they are derived from body water without obvious fractionation.[11,19,26,27] The $\delta^{18}O$ of other output fluxes subjected to fractionation (CO_2 production, O incorporated into organic products, urea, oral and nasal respiration, and transcutaneous water vapor) can be expressed as $\delta_{bw,i} + \varepsilon$, where ε is the isotopic fractionation between an output flux and body water. Hence $\delta_{bw,i}$ is solved

$$\delta_{\text{bw},i} = (M_{\text{input}O}\delta_{\text{input}O} + M_{\text{bw},i-1}\delta_{\text{bw},i-1} - M_{\text{oral}}\varepsilon_{\text{oral}} - M_{\text{nasal}}\varepsilon_{\text{nasal}} - M_{\text{cutan}}\varepsilon_{\text{cutan}} - M_{\text{CO2}}\varepsilon_{\text{CO2}} - M_{\text{p}}\varepsilon_{\text{p}})/(M_{\text{input}O} + M_{\text{bw},i}) \quad (2)$$

Most of the calculations were identical to the MK model by Chen et al.,[20] but some were changed to account for data availability and specific conditions.

Input Fluxes. Air O uptake and water vapor intake were calculated based on body weight, air temperature, and relative humidity. Body weight was set to the average weight of the breeds of each farm and ranged from 430 to 750 kg. The amount of chemically bound O in feed was determined from the energy extraction factor, chemical composition of the feed, digestibility, and dry matter intake. Dry matter intake was calculated from the energy extraction factor, chemical composition of the feed, digestibility, and metabolizable energy demand which was determined by milk production, days in gravidity, and body weight. An average number of days in gravidity (90 d) was used because no farm practiced seasonal calving but all had calves all year round. Feed moisture was assumed to be the sum of adhering water from intercepted rain and dew, and internal water, which was determined from the water contents of maize silage (68 g/g), grass silage (65 g/g), and fresh grass (72 g/g). Adhering water was calculated according to Sun et al.[17] Drinking water intake was estimated according to Cardot et al.[28]

Output Fluxes. The CO_2 production depended on the ingested feed, digestibility, an energy extraction factor, and the amount of O flowing into organic products (milk, growth). The organic products were calculated from milk production and days in gravidity. Respiratory water was estimated from air flow through the lungs, which was influenced by temperature. We assumed that two-thirds of the respiratory water was from oral expiration and one-third was from nasal expiration. Transcutaneous vapor was calculated from ambient conditions (air temperature) and animal surface area. As fecal, urinary, and sweat waters are almost isotopically equal to body water, without obvious fractionation,[20] their amounts were combined and calculated as the difference between total water intake and all other water losses.

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Input Flux $\delta^{18}O$. $\delta^{18}O$ of air O utilized in the lungs was set to a typical value of 15.1‰.[20] For $\delta^{18}O$ of air vapor intake into the lungs, the relationship between average daily temperature and $\delta^{18}O$ of vapor reported by Chen et al.[20] was used. This value had been determined previously on a farm at a location within our research area (average distance from our farms was about 100 km).[29] The $\delta^{18}O$ of tap water was measured. At farms 10 and 11, the drinking water of cows while on pasture was supplied as spring water. The spring water was assumed to have originated from precipitation one month previously. The $\delta^{18}O$ of monthly precipitation was set to the average of the two isotope stations in the respective altitude group of a specific farm (high or low altitude). This precipitation value was also used for the $\delta^{18}O$ of adhering water.

The $\delta^{18}O$ of feed moisture was determined from a mass balance of internal water and adhering water from rain and dew. The internal water depends on the leaf-to-stem ratio and the $\delta^{18}O$ of stem (δ_{stem}) and leaf water (δ_{leaf}). The δ_{leaf} varies daily and diurnally depending on the weather conditions. Chen et al.[20] had used MuSICA (Multilayer Simulator of the Interactions between a vegetation Canopy and the Atmosphere) to model the $\delta^{18}O$ of leaf water with a resolution of 30 min. MuSICA is demanding in terms of its requirements for spatially accurate soil and weather data[30] and cannot easily be applied to ordinary farms. Data by Chen et al.[20] show that δ_{leaf} is equal to δ_{stem} at sunrise (6:00), then increases to its daily maximum (δ_{leaf_max}) at around 14:00, and returns to δ_{stem} at 24:00. The diurnal course can thus be approximated if δ_{stem} and δ_{leaf_max} are known. δ_{stem} is equal to the water taken up from the soil and was set equal to the mean $\delta^{18}O$ in precipitation of the previous 30 d. For the data obtained by Chen et al.,[20] δ_{leaf_max} correlated with δ_{stem}, average air temperature T_{av}, and relative humidity H ($\delta_{leaf\_max} = 0.218T_{av} - 22.6H + 24.5 + \delta_{stem}$; $R^2 = 0.36$; $P < 0.05$; $N = 1642$). This allowed approximating δ_{leaf_max} for other farms and years.

For pasture grass a ratio leaf:stem of 9:1 and grazing peaks at 05:30, 11:00, 15:30, and 21:30 were assumed according to Chen et al.[20] For part-time grazing, we assumed grazing peaks at 9:00, 11:00, and 15:00. The feed intake during these grazing periods was set equal and was averaged to obtain δ_{leaf} for an individual day. For simplicity, the $\delta^{18}O$ in cut-grass intake was assumed to be the same as that in part-time pasture grass because cutting only took place during daylight. The $\delta^{18}O$ of silage water was a weighted value of maize and grass silage. Exchange with air humidity was considered according to Sun et al.[13] assuming an exposure of the silage to air for 24 h.

In the MK model, $\delta^{18}O$ of chemically bound O in pasture or cut grass is assumed to be the same as that in cellulose although some variation in $\delta^{18}O$ exists among different compounds in plant material.[31] The $\delta^{18}O$ of chemically bound O was estimated using the $\delta^{18}O$ of stem water and leaf water during the previous 30 d and a constant fractionation associated with cellulose synthesis. The $\delta^{18}O$ of cellulose in silage (or hay) was assumed to be equal to the average growing-season cellulose O for all farms feeding fresh grass. For the remaining farms, feeding a total mixed ration based on silage, no calculation of leaf and stem water was necessary. In these three cases, which were all close to the farm analyzed by Chen et al.[20] (mean distance 23 km), the $\delta^{18}O$ of cellulose as determined by these authors was used.

Output Flux $\delta^{18}O$. The $\delta^{18}O$ of output fluxes was determined from δ_{bw} and the specific fractionation values between output fluxes and body water according to the MK model.[20]

Statistics. Data are presented as mean value ± the standard deviation. Related measures like the 95% interval of confidence of the mean or the 80% interval of the population are also given where appropriate. Data were evaluated in R software (version 3.3.1) by linear, quadratic, and multiple regressions. The best model of multiple regressions was chosen according to the lowest value of the Akaike information criterion. Significance was defined as $P < 0.05$. Root mean squared error (RMSE) was used to quantitate the deviations between modeled and measured values.

RESULTS AND DISCUSSION

Measurements. *Seasonal Variation in δ_{milk}.* The δ_{milk} ranged from −2 to −10‰ with higher values in summer than in winter (Figure 3). The especially high values in summer

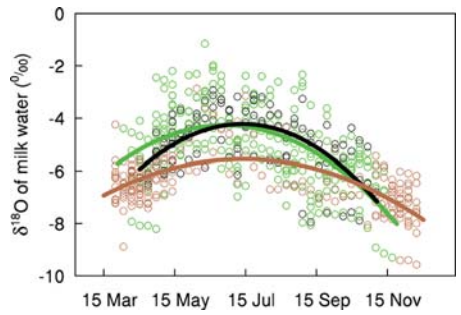

Figure 3. Seasonal variation of $\delta^{18}O$ in milk water depending on feeding strategies of pasture grass (green), cut grass (black), and no grass (brown). Solid lines represent quadratic regressions.

were associated with the pasture grass strategy, while the low values in winter coincided with the strategy of no grass. There was no significant difference ($P > 0.05$) of the quadratic regressions between the pasture grass and cut grass strategies although the data for the pasture grass strategy were more scattered than those for the cut grass strategy. Additionally, the quadratic regressions for fresh and cut grass strategies were both significantly different ($P < 0.05$) from that for the no-grass strategy, which exhibited a smaller increase during summer than the two other strategies.

$\delta^{18}O$ of Drinking Water. Tap water generally varied little between farms (SD: 0.5‰) with an average of −10.8‰. The water extracted near the downstream shoreline of a lake was more enriched (−9.4‰) while the paleo-water was more depleted (−11.5‰) than average tap water.

$\delta^{18}O$ of Silage Water. $\delta^{18}O$ values of grass silage and maize silage were clearly and highly significantly different (Table 1)

Table 1. Sample Size (N) and $\delta^{18}O$ Values of Grass Silage and Maize Silage and 95% Interval of Confidence of the $\delta^{18}O$ Mean (95% CI) and 80% Interval of the $\delta^{18}O$ Population

silage type	N	$\delta^{18}O$ mean (‰)	$\delta^{18}O$ 95% CI (‰)	$\delta^{18}O$ 80% interval (‰)	mean $\delta^{2}H$ (‰)
maize	18	−6.48	±0.39	±1.04	−66.08[b]
grass[a]	42	−10.89	±1.79	±7.48	−101.80[b]

[a]Values of grass silage include data from Sun et al.[13] [b]Mean $\delta^{2}H$ is given for sake of completeness but is not used here.

with $\delta^{18}O$ of maize silage being 4‰ higher than that of grass silage. Also, grass silage was very variable while maize silage covered a narrow range (80% interval of the population on Table 1) although both silages were taken from a similar number of farms and at different times.

Feed Composition and Milk Yield. The average dry-weight proportions of concentrate feed, conserved grass (silage or hay), maize silage, cut grass, and pasture grass for all the 28 farms in the sample were 12%, 49%, 7%, 10%, and 22%, respectively, but there were large differences between farms and seasons (Figure 2). Energy corrected milk yield also covered a large range and varied between 9 and 31 kg d^{-1}.

Influence of Feed Components on δ_{milk}. In the warm season, δ_{milk} decreased significantly with increasing percentage

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of grass silage and hay in the diet ($P < 0.05$; Figure 4B). The farms supplied with lake water (farms 6, 27, and 28), the $\delta^{18}O$ value of which was 1.4‰ higher than average tap water, also had significantly higher δ_{milk} (0.5‰) at the same content of silage and hay. Farm 12 supplied with paleo-water (0.7‰ more negative) also had lower δ_{milk} than average. In this case, the effect became more pronounced when there was less grass in the diet, because this farm replaced grass by hay and thus also the amount of drinking water increased with increasing proportion of hay intake. The effects related to grass intake were opposite to those for grass silage and hay intake (Figure 4A). Concentrates or maize varied less in amount and had only a small influence on δ_{milk} during the warm season (0.9‰; Figure 4C,D) and none in the cold season. In the cold season, average δ_{milk} was lower than that in the warm season. Due to the influence of ambient conditions, this was even true for farms that did also not use fresh grass in the warm season (−6.8 ± 0.8‰ vs −5.6 ± 1.0‰).

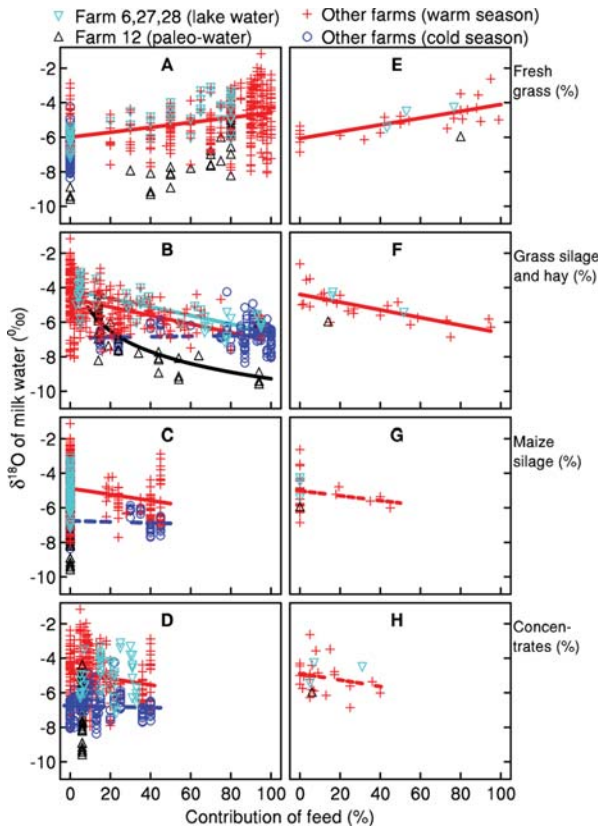

Figure 4. Relationships between $\delta^{18}O$ of milk water and feed content (% dry feed). Crosses and circles represent warm and cold seasons. Fresh grass includes pasture and cut grass. Solid lines represent significant ($P < 0.05$) regressions; dashed lines are not significant. Regressions for farms 6, 27, and 28, which used lake water, and for farm 12, which used paleo-water, are only shown in panel B for clarity. Panels A–D show all data, while panels E–H only display May data.

The scatter was rather large due to the influences of ambient conditions. When the data were restricted to a specific month (May) with the same large variation in the contributions of different feed components to dry matter intake (fresh grass: 0 to 99%; grass silage and hay: 1 to 95%; maize silage: 0 to 40%, concentrates: 0 to 40%,) but in which the ambient condition were less variable, correlations became much closer (Figure 4E–H). The relation with fresh grass percentage had a $R^2 = 0.50$ and that with grass silage and hay percentage had a $R^2 = 0.47$. The change in δ_{milk} was only 2‰, while the $\delta^{18}O$ of grass water and silage water differed by 4‰. This indicated that the effect of feed was reduced by the influence of other fluxes.

Influence of Ambient Conditions on δ_{milk}. High temperature was associated with enriched ^{18}O in milk water. The linear relationships between temperature and δ_{milk} were significant ($P < 0.05$) for all feeding strategies (Figure 5). Regressions between temperature and δ_{milk} in the cut grass and pasture grass strategies overlapped and were significantly different from that in the no-grass strategy. Low relative humidity came along with enriched ^{18}O in milk water. The regression between humidity and δ_{milk} was only significant for the no-grass strategy and the pasture grass strategy ($P < 0.05$). The regressions for pasture grass strategies were significantly different from that for the no-grass strategy. $\delta^{18}O$ of monthly precipitation was significantly ($P < 0.05$) and positively related to that of milk in all strategies (Figure 5). Pasture grass and cut grass strategies exhibited almost the same linear regressions, while the no-grass strategy caused lower values. The relationships for the cut grass and the pasture strategies can only partly be attributed to the rain signal entering the grass by root uptake and adhering water because the relation was also significant, although significantly flatter, for the no-grass strategy. Hence ambient conditions (such as temperature and humidity) must also play a role.

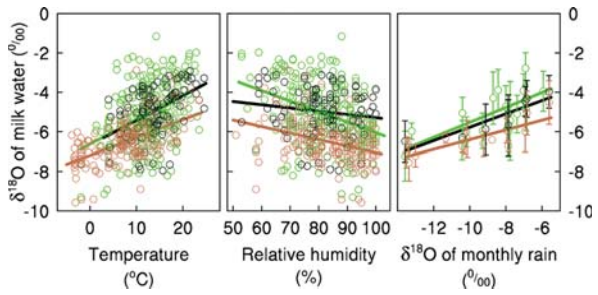

Figure 5. Relationships between the $\delta^{18}O$ of milk water and air temperature, relative humidity and $\delta^{18}O$ of monthly mean of precipitation for pasture (green), cut grass (black), and no grass (brown) strategies. Lines represent the linear regressions (all relations were significant except that for humidity and the cut grass strategy). Error bars in right panel display SD within one month and one type of altitude.

Combined Influences of Feeding Strategy, Ambient Conditions, and Drinking Water. A multiple regression between δ_{milk} and relative humidity H, average daily air temperature T_{av} (°C), grass content C_g in dry feed (%), and $\delta^{18}O$ of drinking water δ_{dw} (‰) was highly significant ($P < 0.001$; $R^2 = 0.56$):

$$\delta_{milk} = -1.0 - 1.9H + 0.066T_{av} + 0.014C_g + 0.42\delta_{dw} \quad (3)$$

The multiple regression in eq 3 indicated positive influences of grass content, temperature, and $\delta^{18}O$ of drinking water and a negative influence of humidity. Temperature had the largest effect and explained about 2‰, while each of the other parameters explained about 1‰.

The seasonal variation in δ_{milk} was 8 ‰. The same variation could be induced by a 9‰ variation of annual precipitation.[8] This suggests that the geographical information may be easily masked by variation caused by season. Also production

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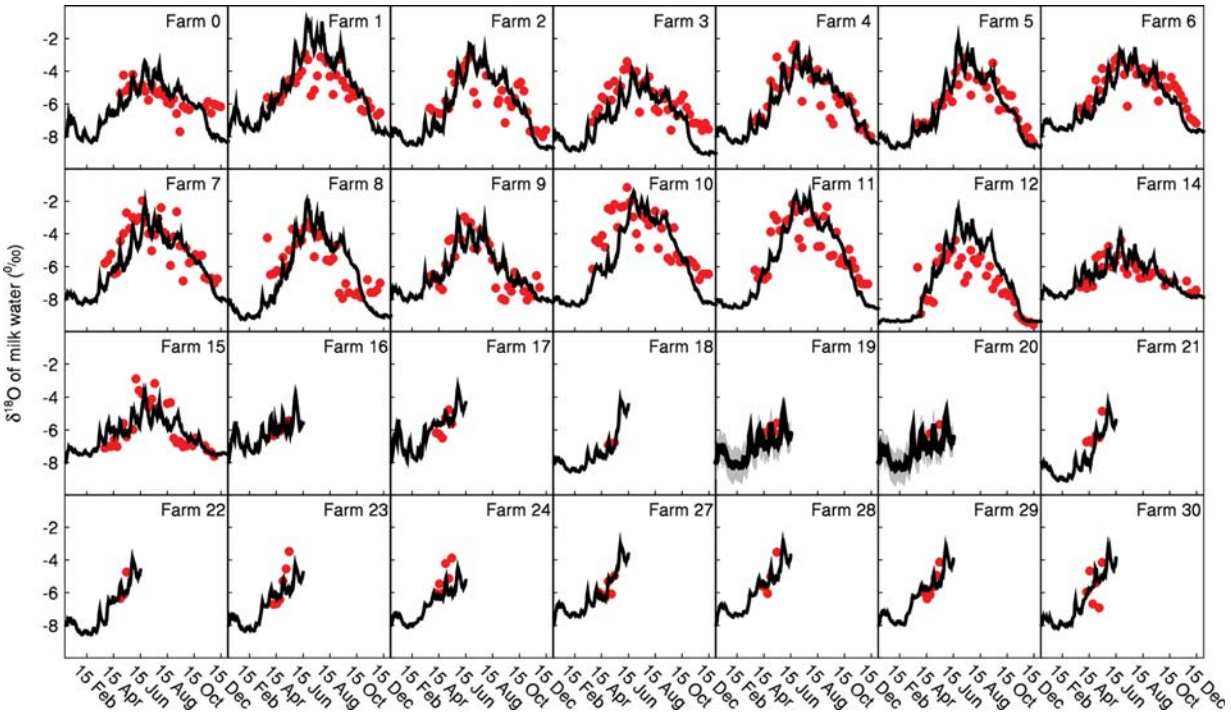

Figure 6. Measured (red circles) and modeled (black lines) $\delta^{18}O$ of milk water in different farms. Shaded areas of farms 19 and 20, which almost entirely fed grass silage, display the range that results when the $\delta^{18}O$ of grass silage varies within the 80% interval of the measured values.

conditions were masked by the seasonal variation. A consideration of the month of production (this information is known for many dairy products) improved the separation between production systems considerably, as indicated by the much closer correlation if only the data of one month were used. The same likely would be true for the geographical information. The influence of production system was largest in the warm season when it caused a difference in δ_{milk} of about 2‰, while feeding and δ_{milk} differed little during the cold season. Distinguishing between production systems may only be possible in situations where both the season and location are known.

Modeling. Modeling predicted a large fluctuation within short periods of time (Figure 6) due to changes in ambient conditions. Nevertheless, the modeled δ_{milk} reflected the seasonal variation and the variation among farms well. The RMSE between modeled and measured data was 1.1‰ while the measured values covered a total range of 8.4‰. In summer, milk was more enriched than in winter. Even when the same type of feed was supplied throughout the year (farms 14 and 15) there was still a seasonal variation in measured and modeled data but the variation of δ_{milk} was smaller (SD of modeled values: 1.1‰; SD of measured values: 1.1‰) than that for the other farms (SD modeled: 1.7‰; SD measured: 1.5‰) because the other farms were subject to seasonal influences of both ambient conditions and changing feed.

The farms that used maize silage in addition to grass products (farms 0, 14, 15, and 16), which involved a high contribution of concentrates, exhibited a lower seasonal variation in measured and modeled values than other farms. Modeling showed that this was due to the large input of feed that did not vary strongly in $\delta^{18}O$ (maize silage, concentrates, and drinking water). Milk yield did not influence the variation. Farm 3 (mean energy corrected milk yield: 24 kg/d/cow) and farm 4 (mean energy corrected milk yield: 14 kg/d/cow), which both used similar feed sources (>80% pasture grass during summer), exhibited very similar absolute values and similar seasonal variation in δ_{milk}. Modeling showed that milk yield had little influence because milk yield influenced the intake of leaf water high in $\delta^{18}O$ and drinking water low in $\delta^{18}O$ to the same degree.

Measurements and modeling showed that influences on $\delta^{18}O$ of body water and derived products are much more complicated, and they are affected by many more conditions of animal environment and husbandry than the influences on $\delta^{13}C$ and $\delta^{15}N$, which are also used to identify production conditions.[32−34] Therefore, it required a highly sophisticated model with demanding data input. We were able to replace some of the input data that were used previously.[20] In particular, these were the data needed to feed the MuSICA model for estimating leaf water and chemically bound O. Replacing MuSICA by a simpler approach also allowed application of the MK model for commercial farms for which soil hydrology data and half-hourly weather data are not usually available and laborious to determine.

The MK model estimated the seasonal variation of δ_{milk} generally well, but despite the sophisticated model and high data input the predictions did not perform better than a simple multiple regression. A regression cannot be applied to situations apart from those covered by the input data. While we covered the entire year and a quite wide range of production systems, our regional scale was small compared to the scale at which $\delta^{18}O$ in precipitation usually changes.[35] It is questionable whether seasons or production systems have the same effect on δ_{milk} as geographic influences like the latitudinal or the continental effects.[8] Hence our regression, as well as other regressions reported in the literature, is of limited value for prediction purposes. The MK model does not have this limitation and thus is superior to multiple regressions although its performance is far from perfect. The reasons for the

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deviations between predictions and measurement are manifold but four main reasons of uncertainty can be identified:

Precipitation. Among meteorological parameters, precipitation is especially difficult to estimate because it may change considerably within a few kilometers[36] or even within a kilometer.[37] This is especially true for high intensity rainfall events. These rains differ also isotopically from average precipitation due to the amount effect.[38] The small-scale pattern of thunderstorm cells thus not only influences water availability and in turn leaf water enrichment[39] and the amount of adhering rain and dew water,[17,40] but it also influences the $\delta^{18}O$ of soil water for a considerable period of time until enough subsequent rain has fallen to level out the effect of a large individual rainfall event. Also the temporal variation is large. The mean standard deviation for rain events within one month at one site was 3.5 ‰ (unpublished data) indicating that the 95% interval for events occurring in one month could cover a range of 14 ‰. These spatiotemporal uncertainties restrict predictions but also the improvement of the model. For the latter, measurements of rain and also air vapor composition, which we estimated from rain, should be measured for improving the model instead of using published sources.

Farmer. The daily workflow of a farmer changes between seasons or even from day to day. If herbage is cut very early in the day the leaf water will be similar to the soil water, whereas herbage cut in the early afternoon will have leaf water close to maximum enrichment.[20,41] This influence will be especially large in cut-grass systems where a large share of feedwater intake is subject to these individual decisions. The large fluctuations between subsequent measurements, which were not related to meteorological variation captured by the MK model in cut-grass systems (farms 1, 6, and 8), likely have been caused by these decisions.

Animal. The behavior of the animal is not included in the MK model. It will have a large influence especially in pasture systems, in which animals can adapt their grazing behavior according to meteorological conditions like temperature, rain, and wind.[42,43] This behavior could especially be observed in farm 12, where animals had the opportunity to choose between pasture and hay feeding. While they usually showed a preference for grazing pasture herbage, on very hot days they switched to feeding on hay to escape the sun and to increase their intake of cool tap water. Thus, they replaced enriched leaf water by tap water especially on days when the largest enrichments can be expected. Switching to night-time grazing and resting during the day would have a similar effect.

Silage. From Farmer and Animal it follows that the least variation and uncertainty should occur in no-grass systems because of the standardized method used to prepare and provide the feed (especially with total mixed ration).[44] Indeed, seasonal variation was smaller in these systems (farms 14 and 15) but uncertainty remained. In this case, uncertainty can be attributed to a large degree to $\delta^{18}O$ in grass silage water, which contributes a large share to water intake and turned out to be highly variable as shown by the shaded area of farms 19 and 20 in Figure 6. This variability relates to the production of grass silage. Once the grass has been cut, the silage must be produced independent of the weather. In hot weather and on dry soil, enrichment by evaporation will prevail and cause enrichment of the residual water. With rainy weather and high soil moisture, isotopic exchange of the cut grass on the soil surface and soil water will occur[13] and cause low $\delta^{18}O$ in silage water. Even different portions of grass silage produced during a day may differ. The differences cannot be predicted when the silage is fed. Maize silage, in contrast, always had high $\delta^{18}O$ values with little variation, because maize is not dried on the ground where exchange with soil water may occur. Furthermore, there is no need to harvest maize under rainy weather but harvest can be delayed until the standing crop has dried by evaporation to the desired moisture content. As a consequence, the isotopic difference between grass silage and maize silage was large and covered the entire range found in feed. It was surprising that no data for maize silage water were available although maize silage can contribute a large share to cattle feed of up to 80% under regional conditions.[23]

Previously, $\delta^{18}O$ in milk water and other animal products has been related to the regional variation of $\delta^{18}O$ in precipitation or tap water.[8] Our study showed that this relation also exists for the relation between the seasonal variation in precipitation and in milk but it cannot exist for tap water, which usually is constant throughout the year. The relation between $\delta^{18}O$ in precipitation and in animal water may be interpreted as a causal relation but our modeling showed that the reason for similar seasonality is that both parameters are influenced by temperature. For precipitation this influence results from the temperature influence on equilibrium fractionation and the rain out,[45] while in the case of animal body water the mechanisms are more complex: Temperature will additionally influence evaporative enrichment in leaf water and the evaporative enrichment in the animal. In consequence of two enrichments adding onto the seasonal variation in precipitation, a larger effect in milk than in precipitation should result. This was not the case because drinking water intake usually also increases with increasing temperature and counteracts the aforementioned enrichments. These complex interactions can easily be overlooked in regression analyses, while temperature enters the MK model in many places illustrating its manifold influences.

Tap water varied only little in $\delta^{18}O$ but this effect was still important due to its large and consistent contribution to dietary water intake. It is usually assumed that tap water is close to mean precipitation.[46] Our study showed that this is not necessarily the case. Even neighboring farms receiving the same precipitation had differences in tap water when supplied from different water works. Also the frequency of farms receiving lake water was larger than expected, and especially in mountainous areas, where fractured bedrock aquifers dominate, lake water is frequently used and may be pumped over distances in the range of 100 km. In such cases, the tap water will be considerably above mean local precipitation because it originates from low altitude and is further enriched in ^{18}O due to lake evaporation.

Dairy cows vary considerably in milk production due to differences in breed, stage of lactation, feeding, and other production variables. Our data set covered a wide range in herd milk yield (9−31 kg/d/cow) and an even wider range for individual cows but milk yield did not enter the multiple regression. The MK modeling showed that an increasing intake of feed high in $\delta^{18}O$ to produce more milk also increases the intake of tap water low in $\delta^{18}O$. Both changes are tightly coupled and level out. This also justifies using an animal model (the MK model) for modeling tank milk. Milk yield, which varies considerably between cows and stages of lactation, does not have a detectable influence and all other cow-specific parameters (e.g., weight) have only a weak influence and a small range within a herd, while the ambient conditions are

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identical within a herd. The MK model suggested that drinking water sources and feeding components (especially grass and silage content) should be deliberately investigated before using $\delta^{18}O$ as origin tracer. The MK model also hinted that the distinction of different production systems by $\delta^{18}O$ requires knowledge about ambient conditions (especially temperature) which contribute a lot to the isotopic seasonal variation of body water.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02482.

Table A1: Locations of the 28 farms that provided the focus for this study. Table A2: Parameters, abbreviations, and units used in this paper. Table A3: Calculation methods for input fluxes according to the MK model. Table A4: Calculation methods for output fluxes according to the MK model. Table A5: Parameters of regressions shown in figures together with their confidence intervals. Figure A1: Monthly weather and δ^{18} in precipitation in 2005 from meteorological and isotope stations. (PDF)

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Notes

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Supplementary information

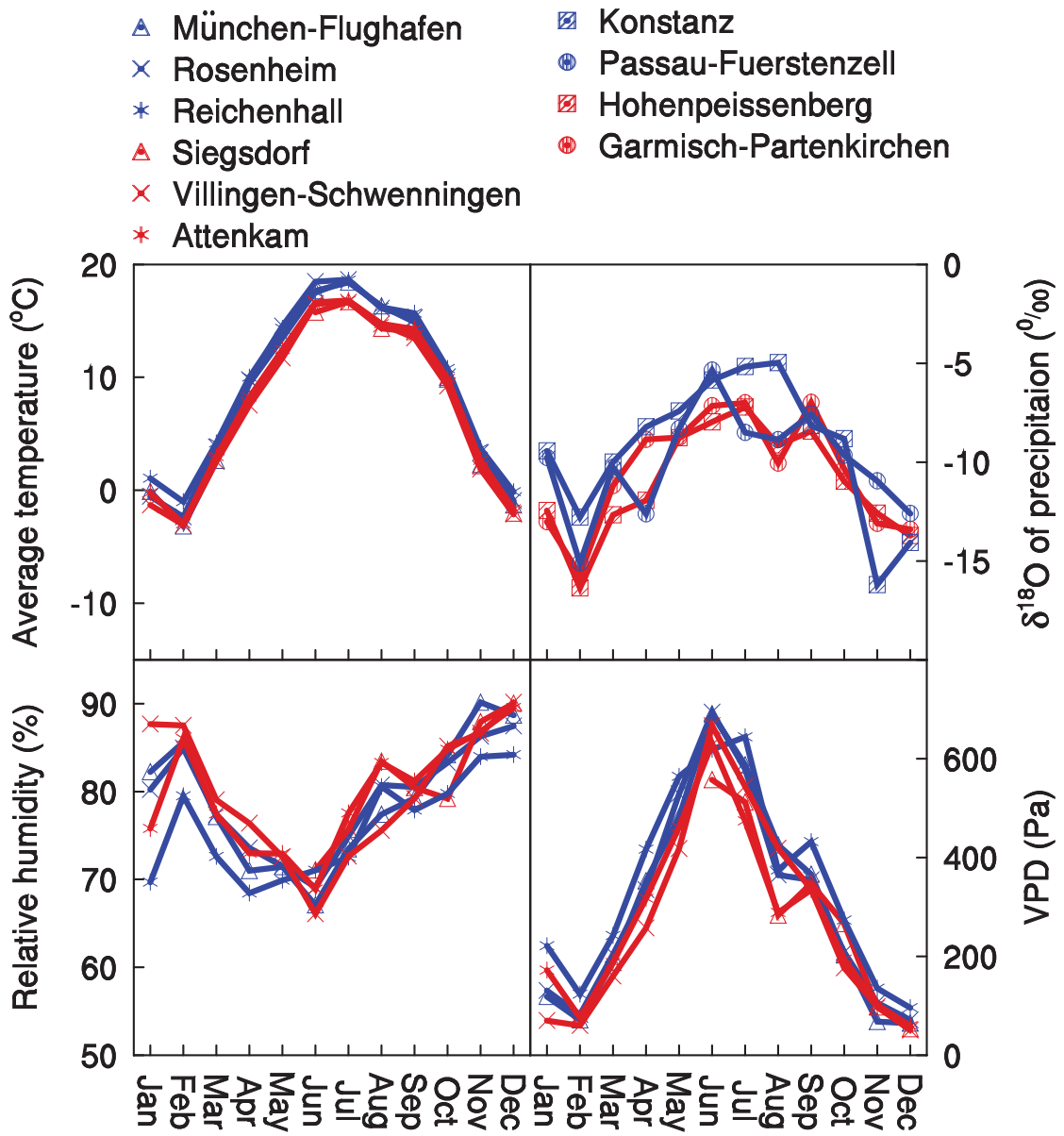

Figure A1: Monthly weather and $\delta^{18}O$ in precipitation in 2005 from meteorological and isotope stations within the research area at high altitude (>600 m; red symbols and lines) and low altitude (<600 m, blue).

Table A1: Locations and Production Conditions of the 28 Farms that Provided the Focus for this Study.

Farm number	Latitude (°)	Longitude (°)	Altitude (m above sea level)	Altitude group	$\delta^{18}O$ of tap water (‰)	Breed	Average milk yield (kg/d)
0	47.86	11.28	635	High	-10.7	Fleckvieh	25
1	48.17	12.73	426	Low	-10.2	Fleckvieh	14
2	47.94	12.60	553	Low	-11.0	Fleckvieh	19
3	47.91	12.82	448	Low	-11.4	Fleckvieh	24
4	47.85	12.82	496	Low	-10.9	Pinzgauer	14
5	47.94	12.01	497	Low	-11.1	Fleckvieh	20
6	47.88	12.34	561	Low	-9.4	Fleckvieh	24
7	47.99	12.18	487	Low	-10.5	Fleckvieh	18
8	47.94	12.01	497	Low	-11.8	Fleckvieh	18
9	47.76	12.65	654	High	-10.2	Fleckvieh	
10	47.71	7.81	503	Low	-10.7	Vorderwälder	19
11	47.75	7.80	691	High	-11.3	Hinterwälder	11
12	48.37	11.72	450	Low	-11.5	Holstein Friesian	20
14	48.17	11.81	515	Low	-10.3	Fleckvieh	26
15	48.36	11.89	447	Low	-9.9	Fleckvieh/Holstein Friesian	25
16	48.17	12.73	426	Low	-10.2	Fleckvieh	21
17	48.06	12.77	384	Low	-10.9	Fleckvieh	
18	48.00	12.64	532	Low	-10.9	Fleckvieh	16
19	48.00	12.64	532	Low	-10.9	Fleckvieh	17
20	47.85	12.82	496	Low	-10.9	Rad Holstein	
21	47.82	12.64	615	High	-12.0	Fleckvieh	16
22	47.84	12.61	579	Low	-11.1	Fleckvieh	19
23	47.80	12.17	469	Low	-10.6	Fleckvieh	
24	47.78	12.21	698	High	-10.4	Fleckvieh	18
27	47.88	12.34	561	Low	-9.4	Fleckvieh	19
28	47.88	12.34	561	Low	-9.4	Fleckvieh	20
29	47.91	12.30	522	Low	-10.2	Fleckvieh	19
30	47.98	12.13	481	Low	-10.3	Holstein Friesian	17

Table A2: Parameters, Abbreviations and Units Used in this Paper; Part 1: Fluxes.

Flux	Abbreviation	Unit
Intake of water adhering to feed	M_{adhere}	mole/d
O intake from air	M_{air}	mole/d
Intake of bound H in digested feed	M_{bH}	mole/d
Intake of bound O in digested feed	M_{bO}	mole/d
Body water	M_{bw}	mole/d
O in carbon dioxide production	M_{CO2}	mole/d
Transcutaneous vapor flux	M_{cutan}	mole/d
Drinking water intake on dry day (when $(P_{i-1} + P_i) < 0.02$ mm)	M_{dry}	mole/d
Drinking water intake	M_{dw}	mole/d
Fecal water loss	M_{fecal}	mole/d
Feed moisture uptake	M_{fw}	mole/d
Nasally exhaled water loss	M_{nasal}	mole/d
Orally exhaled water loss	M_{oral}	mole/d
O flowing into organic products	M_p	mole/d
Exhaled water by respiration	M_{resp}	mole/d
Sweat water loss	M_{sweat}	mole/d
Intake of feed internal water	M_{inner}	mole/d
Total water input flux	$M_{input\ H2O}$	mole/d
Total O input flux	M_{inputO}	mole/d
Total O output flux	$M_{outputO}$	mole/d
Total water output flux	$M_{output\ H2O}$	mole/d
O flux with urea	M_{urea}	mole/d
Urinary water loss	$M_{urinary}$	mole/d
Vapor O uptake by breathing	M_{vapor}	mole/d
Drinking water intake on wet day (when $(P_{i-1} + P_i) > 2$ mm)	M_{wet}	mole/d

Table A2, Continued: Parameters, Abbreviations and Units Used in this Paper; Part 2: Isotope Compositions.

Water or O source	Abbreviation	Unit
Grass silage water before exposure to air	δ_{gs_0}	‰
Maize silage water before exposure to air	δ_{ms_0}	‰
Water adhering to feed	δ_{adhere}	‰
O utilized in lungs	δ_{air}	‰
Body water	δ_{bw}	‰
Cellulose	δ_{c}	‰
Bound O in feed	δ_{bO}	‰
Drinking water	δ_{dw}	‰
Feed moisture	δ_{fw}	‰
Total O input flux	δ_{inputO}	‰
Leaf water intake by cows	δ_{leaf}	‰
Max value of leaf water within one day	δ_{leaf_max}	‰
Milk water	δ_{milk}	‰
Nasally exhaled water	δ_{nasal}	‰
Orally exhaled water	δ_{oral}	‰
Total O output flux	$\delta_{outputO}$	‰
O flowing into organic products	δ_{p}	‰
O in monthly precipitation	δ_{precip}	‰
Precipitation of the previous 30 d	$\delta_{precip,30}$	‰
Silage water in equilibrium with air	δ_{ss}	‰
Stem water	δ_{stem}	‰
Transcutaneous vapor	δ_{tran}	‰
O flux with urea	δ_{urea}	‰
Vapor in free air	δ_{vapor}	‰

Table A2, Continued: Parameters, Abbreviations and Units Used in this Paper; Part 3: ^{18}O Fractionations.

Fractionation	Abbreviation	Unit
between CO_2 and body water	ε_{CO2}	‰
between transcutaneous vapor and body water	ε_{cutan}	‰
between vapor and water in equilibrium	ε_{eq}	‰
kinetic fractionation	ε_{k}	‰
between nasally exhaled water and body water	ε_{nasal}	‰
between oral exhaled water and body water	ε_{oral}	‰
between organic products and body water	ε_{p}	‰
between carbonyl O and water	ε_{wc}	‰

Table A2, Continued: Parameters, Abbreviations and Units Used in this Paper; Part 4: Other Parameters.

Parameter	Abbreviation	Unit
Air flow through the lungs	A	L/d
Oxygen content in air (21%)	C_{air}	%
Dry matter content of carbohydrates in feed	C_c	%
Dry matter content of concentrates in feed	C_{concen}	%
Dry matter content of fat in feed	C_f	%
Dry matter content of fresh grass in dry feed	C_g	%
Dry matter content of maize in dry feed	C_{ms}	%
Dry matter content of crude protein in feed	C_p	%
Dry matter content of grass silage and hay in dry feed	C_{sh}	%
O conversion factor (0.00216 mole/KJ)	c_o	mole/KJ
Digestibility	D	
Days in gravidity	d_g	d
Day of year	d_y	d
Energy extraction efficiency	E_{ex}	
Energy used for heat production	E_H	KJ/d
Metabolizable energy	E_{met}	KJ/d
Energy used for mass production	E_P	KJ/d
Fresh matter fraction of fresh grass in feed	F_g	
Fresh matter fraction of grass silage in feed	F_{gs}	
Fresh matter fraction of hay in feed	F_h	
Fresh matter fraction of maize silage in feed	F_{ms}	
Unit conversion factor L → mole (55.56 mole/L)	f_{mL}	mole/L
Unit conversion factor d →s (86400 s/d)	f_{sd}	s/d
Unit conversion factor m^3 →mole (3 mole vapor/m^3 at body temperature)	f_{mcm}	mole/m^3
Unit conversion factor d →min (1440 min/d at body temperature)	f_{mind}	min/d
Unit conversion factor kJ →Ws (1000 Ws/kJ)	f_{WskJ}	Ws/kJ
Relative humidity	H	
Animal mass	m_{animal}	kg
Dry mass intake of feed	m_{dry}	kg/d
Milk production	m_{milk}	kg/d
Oxygen extraction from air (0.2)	O_{ex}	
Proportion of O atoms exchanging with medium water during cellulose synthesis	P_{ex}	
Precipitation at day i	P_i	mm/d
Relative plant available water	P_{rel}	mm/mm
Proportion of (unenriched) source water in tissue where cellulose synthesis is occurring	P_x	
Leaf to shoot ratio of feed	R	kg/kg
Fraction of adhering water in feed water	R_{adhere}	kg/kg
Fraction of grass water in feed water	R_g	kg/kg

Fraction of grass silage water in feed water	R_{gs}	kg/kg
Fraction of maize silage water in feed water	R_{ms}	kg/kg
Animal surface area	S	m^2
Half-life of silage water	$t_{0.5}$	h
Exposure time of winter feed	$t_{exposed}$	h
Average daily temperature	T_{av}	°C
Minimum daily temperature	T_{min}	°C
Vapor pressure deficit	VPD	Pa
Molar gas volume (25.5 L/mole at 38 °C)	V_m	L/mole
Water content of fresh feed	W_C	g/g
Water content of fresh grass	W_g	g/g
Water content of grass silage	W_{gs}	g/g
Water content of hay	W_h	g/g
Water content of maize silage	W_{ms}	g/g

Table A3: Calculation Methods for Input Fluxes according to the MK Model[20]. Modifications to meet specific requirements of this data set are indicated by superscripts after the parameter and explained below.

Parameter	Function
A	$E_H \times c_o \times V_m / O_{ex} / C_{air} \times 100 =$ $E_H \times c_o \times 607$
E_H	$(5.6 \times m_{animal}^{0.75} + 1.6 \times 10^{-5} \times d_g^3 + 22 \times m_{milk}) \times f_{sd} / f_{WskJ}$ $= 484 \times m_{animal}^{0.75} + 1.4 \times 10^{-3} \times d_g^3 + 1900 \times m_{milk}$
E_P	$2 \times (1.4 \times 10^{-3} \times d_g^3 + 1900 \times m_{milk})$
E_{met}	$E_H + E_P$
m_{dry}	$E_{met} / (170 \times C_c + 400 \times C_f + 200 \times C_p) / D / E_{ex}$
M_{air}	$2 \times C_o \times E_H$
M_{bH}	$2 \times D \times E_{ex} \times m_{dry} \times (0.31 \times C_c + 0.6 \times C_f + 0.11 \times C_P)$
M_{bO}	$2 \times D \times E_{ex} \times m_{dry} \times (0.15 \times C_c + 0.02 \times C_f + 0.03 \times C_P)$
M_{fw}	$m_{dry} \times f_{mL} \times W_C / (1 - W_C) + M_{adhere}$
M_{dw} [#]	$(1.53 \times m_{dry} + 1.33 \times m_{milk} + 89 \times (1 - W_C) + 0.57 \times T_{min} - 0.3 \times P_i - 25.65) \times f_{mL}$ $= 85 \times m_{dry} + 74 \times m_{milk} - 4953 \times W_C + 32 \times T_{min} - 17 \times P_i + 3525$
$M_{input\ H2O}$	$M_{dw} + M_{fw} + M_{vapor} + M_{bH} / 2 - 2 \times M_{urea}$
M_{inputO}	$M_{dw} + M_{fw} + M_{vapor} + M_{bO} + M_{air}$
M_{vapor}	$10^{(0.686+0.027Tav)} \times H \times A / 760 / V_m$
W_C [##]	$F_g \times W_g + F_{gs} \times W_{gs} + F_{ms} \times W_{ms} + F_h \times W_h$
δ_{fw} [###]	$R_g \times [(\delta_{leaf} \times R + \delta_{stem} \times (1 - R)] + \delta_{adhere} \times R_{adhere} + R_{ms} \times [\exp(-0.69 \times t_{exposed} / t_{0.5}) \times (\delta_{ms\_0} - \delta_{ss}) + \delta_{ss}] + R_{gs} \times [\exp(-0.69 \times t_{exposed} / t_{0.5}) \times (\delta_{gs\_0} - \delta_{ss}) + \delta_{ss}]$
δ_{leaf} [†]	Pasture grass strategy: $0.25 \times \delta_{stem} + 0.25 \times [\delta_{stem} + 5/8 \times (\delta_{leaf\_max} - \delta_{stem})] + 0.25 \times [\delta_{leaf\_max} - 0.15 \times (\delta_{leaf\_max} - \delta_{stem})] + 0.25 \times [\delta_{leaf\_max} - 0.75 \times (\delta_{leaf\_max} - \delta_{stem})]$ $= 0.57 \times \delta_{stem} + 0.43 \times \delta_{leaf\_max}$ Cut grass strategy: $1/3 \times [\delta_{stem} + 3/8 \times (\delta_{leaf\_max} - \delta_{stem})] + 1/3 \times [\delta_{stem} + 5/8 \times (\delta_{leaf\_max} - \delta_{stem})] + 1/3 \times [\delta_{leaf\_max} - 0.1 \times (\delta_{leaf\_max} - \delta_{stem})]$ $= 0.37 \times \delta_{stem} + 0.63 \times \delta_{leaf\_max}$
δ_{leaf_max} [††]	$0.218 \times T_{av} - 22.6 \times H + 24.5 + \delta_{stem}$
δ_{ss}	$[\delta_{vapor} + \varepsilon_{eq} / H + \varepsilon_k / H - \varepsilon_k] / [1 + \varepsilon_k / 1000 - \varepsilon_k / 1000 / H - \varepsilon_{eq} / 1000 / H]$
δ_{stem} [†††]	$\delta_{precip,30}$
δ_{vapor}	$0.34 \times T_{av} - 21.52$
δ_c	$(\delta_{leaf} - \delta_{stem}) \times (1 - P_x \times P_{ex}) + \delta_{stem} + \varepsilon_{wc}$ $= 0.58\ \delta_{leaf} + 0.42\ \delta_{stem} + 27$
δ_{inputO}	$[M_{air} \times \delta_{air} + M_{bO} \times \delta_c + (M_{inner} + M_{adhere}) \times \delta_{fw} + M_{dw} \times \delta_{dw} + M_{vapor} \times \delta_{vapor}] / M_{inputO}$

#: according to Cardot et al.[28]

##: maize silage not relevant in Chen et al.[20] was considered explicitly here

###: The mixture of grass water, adhering water, grass silage water, and maize silage water.

†: leaf water in feed was related to the $\delta^{18}O$ of maximum and leaf water and stem water and time of feeding.

††: calculated from the data in Chen et al.[20]

†††: the stem water, directly measured in Chen et al.[20], was assumed equal to the precipitation of the previous 30 d.

Table A4: Calculation Methods for Output Fluxes according to the MK Model.[20]

Parameter	Function
M_{CO2}	$M_{outputO} - M_{output\ H2O} - M_{urea} - M_p$
M_{cutan}	$85.18 \times \exp[(T_{av} - 24.92) / 7.96] \times f_{sd} / (2500.7879 - 2.3737 \times T_{av}) \times S / 18$ $= [\exp(T_{av} / 7.96 + 9.79)] / (2500.7879 - 2.3737 \times T_{av}) \times S$
$M_{fecal}+M_{urinary}+M_{sweat}$	$M_{output\ H2O} - M_{milk} - M_{cutan} - M_{oral} - M_{nasal}$
M_p	$1.8 \times m_{milk} + 5.2 \times 10^{-7} \times d_g^3$
M_{resp}	$f_{mcm} \times f_{mind} \times 0.0189 \times \exp[0.537 \times (2.966 + 0.00069 \times T_{av}^2 + 0.0218 \times T_{av})]$ $= \exp(6 + 0.00037 \times T_{av}^2 + 0.0117 \times T_{av})$
M_{oral}	$2/3 \times M_{resp}$
M_{nasal}	$1/3 \times M_{resp}$
M_{urea}	$m_{dry} \times D \times E_{ex} \times C_p \times 0.06$
S	$0.09 \times m_{animal}^{0.67}$

Table A5: Parameters of Regressions Shown in Figures Together with Their Confidence Intervals (±).

Equation	Figure	N	R^2	P [a]	Subset	Farms
$\delta_{milk} = (-10.0 \pm 2.9) + (6.3 \pm 1.7) \times 10^{-2} \times d_y + (-1.7 \pm 0.4) \times 10^{-4} \times d_y^2$	3	290	0.33	*	Pasture grass	All
$\delta_{milk} = (-12.8 \pm 2.9) + (8.7 \pm 2.6) \times 10^{-2} \times d_y + (-2.2 \pm 0.6) \times 10^{-4} \times d_y^2$	3	107	0.36	*	Cut grass	All
$\delta_{milk} = (-9.3 \pm 0.8) + (3.8 \pm 1.0) \times 10^{-2} \times d_y + (-9.6 \pm 2.2) \times 10^{-4} \times d_y^2$	3	211	0.36	*	No grass	All
$\delta_{milk} = (-6.1 \pm 0.3) + (2.5 \pm 0.6) \times 10^{-2} \times C_g$	4	49	0.57	*	Both seasons	6,27,28
$\delta_{milk} = (-9.3 \pm 0.9) + (4.0 \pm 1.3) \times 10^{-2} \times C_g$	4	37	0.52	*	Both seasons	12
$\delta_{milk} = (-6.0 \pm 0.3) + (1.4 \pm 0.4) \times 10^{-2} \times C_g$	4	379	0.12	*	Warm season	Not 6,12,27,28
$\delta_{milk} = (-5.2 \pm 0.7) + (0.8 \pm 3.4) \times 10^{-2} \times C_{concen}$	4	49	0.004	ns	Both seasons	6,27,28
$\delta_{milk} = (-2.5 \pm 13.6) + (-0.7 \pm 2.3) \times C_{concen}$	4	37	0.01	ns	Both seasons	12
$\delta_{milk} = (-4.8 \pm 0.2) + (-1.8 \pm 1.2) \times 10^{-2} \times C_{concen}$	4	379	0.02	*	Warm season	Not 6,12,27,28
$\delta_{milk} = (-6.8 \pm 0.2) + (-0.3 \pm 0.9) \times 10^{-2} \times C_{concen}$	4	143	0.003	ns	Cold season	Not 6,12,27,28
$\delta_{milk} = (-4.1 \pm 0.3) + (-2.5 \pm 0.6) \times 10^{-2} \times C_{sh}$	4	49	0.59	*	Both seasons	6,27,28
$\delta_{milk} = (-1.4 \pm 1.5) + (-1.7 \pm 0.4) \times \ln(C_{sh})$	4	37	0.64	*	Both seasons	12
$\delta_{milk} = (-4.7 \pm 0.2) + (-2.5 \pm 0.6) \times 10^{-2} \times C_{sh}$	4	379	0.15	*	Warm season	Not 6,12,27,28
$\delta_{milk} = (-7.0 \pm 0.3) + (0.2 \pm 0.4) \times 10^{-2} \times C_{sh}$	4	143	0.005	ns	Cold season	Not 6,12,27,28
$\delta_{milk} = (-4.9 \pm 0.1) + (-1.7 \pm 1.0) \times 10^{-2} \times C_{ms}$	4	379	0.03	*	Warm season	Not 6,12,27,28
$\delta_{milk} = (-6.8 \pm 0.1) + (-0.3 \pm 0.6) \times 10^{-2} \times C_{ms}$	4	143	0.006	ns	Cold season	Not 6,12,27,28
$\delta_{milk} = (-6.1 \pm 0.5) + (2.0 \pm 0.8) \times 10^{-2} \times C_g$	4	28	0.50	*	May	Not 6,12,27,28
$\delta_{milk} = (-4.9 \pm 0.5) + (-1.7 \pm 3.3) \times 10^{-2} \times C_{concen}$	4	28	0.05	ns	May	Not 6,12,27,28
$\delta_{milk} = (-4.4 \pm 0.4) + (-2.3 \pm 1.0) \times 10^{-2} \times C_{sh}$	4	28	0.47	*	May	Not 6,12,27,28
$\delta_{milk} = (-5.0 \pm 0.4) + (-1.5 \pm 2.8) \times 10^{-2} \times C_{ms}$	4	28	0.04	ns	May	Not 6,12,27,28
$\delta_{milk} = (-0.7 \pm 1.3) + (-5.3 \pm 1.6) \times H$	5	290	0.14	*	Pasture grass	All
$\delta_{milk} = (-3.7 \pm 1.9) + (-1.6 \pm 2.3) \times H$	5	107	0.02	ns	Cut grass	All
$\delta_{milk} = (-3.8 \pm 1.1) + (-3.3 \pm 1.2) \times H$	5	211	0.12	*	No grass	All
$\delta_{milk} = (-6.6 \pm 0.5) + (0.1 \pm 0.0) \times T_{av}$	5	290	0.16	*	Pasture grass	All
$\delta_{milk} = (-6.7 \pm 0.6) + (0.1 \pm 0.0) \times T_{av}$	5	107	0.24	*	Cut grass	All
$\delta_{milk} = (-7.2 \pm 0.2) + (0.1 \pm 0.0) \times T_{av}$	5	211	0.33	*	No grass	All
$\delta_{milk} = (-1.7 \pm 1.9) + (0.38 \pm 0.20) \times \delta_{precip}$	5	16	0.53	*	Pasture grass	All
$\delta_{milk} = (-2.4 \pm 1.5) + (0.34 \pm 0.17) \times \delta_{precip}$	5	8	0.80	*	Cut grass	All
$\delta_{milk} = (-4.0 \pm 1.1) + (0.24 \pm 0.10) \times \delta_{precip}$	5	15	0.67	*	No grass	All

[a] "ns" and "*" represent non-significant and significant (P<0.05) relationship.

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Model explanation of the seasonal variation of $\delta^{18}O$ in cow (*Bos taurus*) hair under temperate conditions

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Oxygen isotopes ($\delta^{18}O$) in animal and human tissues are expected to be good recorders of geographical origin and migration histories. However, seasonal variation of $\delta^{18}O$ may diminish the origin information in the tissues. Here the seasonality of $\delta^{18}O$ in tail hair was investigated in a domestic suckler cow (*Bos taurus*) that underwent different ambient conditions, physiological states, keeping and feeding during five years. A detailed mechanistic model was built to explain this variation. The measured $\delta^{18}O$ in hair significantly related ($p < 0.05$) to the $\delta^{18}O$ in meteoric water in a regression analysis. Modelling suggested that this relation was only partly derived from the direct influence of feed moisture. Ambient conditions (temperature, moisture) also affected the animal itself (drinking water demand, transcutaneous vapor etc.). The clear temporal variation thus resulted from complex interactions with multiple influences. The twofold influence of ambient conditions via the feed and via the animal itself is advantageous for tracing the geographic origin because $\delta^{18}O$ is then less influenced by variations in moisture uptake; however, it is unfavorable for indicating the production system, e.g. to distinguish between milk produced from fresh grass or from silage. The model is versatile but needs testing under a wider range of conditions.

The potential use of the $^{18/16}O$ isotope ratio values ($\delta^{18}O$) in animal tissues such as nail, hair, bone and feather to track geographic origin, climate and migration has been recognized during recent decades[1,2]. It is based on empirical correlations between $\delta^{18}O$ in animal tissues and the $\delta^{18}O$ of amount-weighted mean annual (or mean-growing season) precipitation[3]. These correlations have been used to provide authentication of meat and dairy products[4–6] and as tools for use in anthropology and archeology[7]. Among the different types of animal tissues, hair is preferred as the archival record of the animal's diet because it grows continuously and preserves its isotopic information once formed[8]. Significant relations between the $\delta^{18}O$ of tissue and that of precipitation have been found primarily in inorganic molecules such as phosphates and carbonates of bones and tooth enamel; however, an isotopic relation between hair and that of precipitation has not been found for all species (such as felids[3]). Although a general relation between $\delta^{18}O$ in hair and that in precipitation was found for humans[9,10], it is still unknown whether $\delta^{18}O$ in the hair of domestic animals such as cows can be used to track their geographic origin effectively, because their digestive systems differ from that of humans and their feed is also controlled by humans.

The challenge of interpreting $\delta^{18}O$ in hair of domestic animals stems from several reasons. Unlike nitrogen and carbon, O has multiple input fluxes (air O, inhaled air water vapor, chemically bound O in feed, feed moisture and drinking water) and output fluxes (CO_2 production, fecal water, milk water, exhaled water vapor, sweat water, transcutaneous water vapor, urea or uric acid and urine water) that are largely controlled by animal physiology and by environmental influences other than isotopic composition of meteoric water. Thus, the variation of $\delta^{18}O$ in hair cannot directly reflect the variation of $\delta^{18}O$ in precipitation. For instance, drinking water amount can be influenced by precipitation, temperature, relative humidity and plant available water, and also by intake of feed dry matter, crude protein and Na[11–15], thereby causing a varying contribution of drinking water to total O input flux. The $\delta^{18}O$ of water in grass, which is the main feed of cattle at pasture, changes hourly with ambient conditions[16]. Even $\delta^{18}O$ of silage water varies with the fluctuation of ambient conditions and the change of exposure time[17]. Furthermore, the intercepted rain and dew in the grass can be ingested by grazing animals, which further increases the complexity of the O input of animals[15,18]. This suggests that the $\delta^{18}O$ of daily total feed moisture is a complex variable influenced not only by the ambient conditions but also by feeding time and frequency. The husbandry of domestic animals has additional influences on their diets and water intake. The feeding strategy

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changes with the season: in temperate latitudes fresh grass is usually fed in the warm season, which is the main growing period, whereas silage and hay are provided in winter; these sources differ in $\delta^{18}O$ for both organic O and water O. Moreover, husbandry and feeding strategy will differ with production type and intensity (e.g., dairy vs beef; different milk yields among dairy systems). Finally, $\delta^{18}O$ in hair is thought to be derived from exchange of amino-O with gut water[9]. Cows have a four-compartment stomach involved in water absorption and remixing; this makes the prediction of gut water more complicated than for monogastric animals. For these reasons, there is a need for a mechanistic, isotope-enabled $\delta^{18}O$ model related to ambient conditions, and animal husbandry, including feeding and animal physiology, to examine the influence of different parameters on the seasonality of $\delta^{18}O$ in hair.

Several models have described the $\delta^{18}O$ of body water based on the isotopic balance of O[19–23]. However, these models did not link ambient conditions (such as temperature and humidity) and body water together, and they did not consider the influence of ambient conditions on the amount of O input fluxes. Kohn[2] developed a general model to describe the relationships between $\delta^{18}O$ of body water and the $\delta^{18}O$ input fluxes (air O uptake, air water vapor into the lungs, chemically bound O in feed, feed moisture and drinking water) and output fluxes (CO_2 production, fecal water, respiratory water, sweat water, transcutaneous water vapor, urea and urine) based on the isotopic balance and amount balance of O and the influence of ambient conditions. This model was used to analyze the sensitivity of climatic variation and species-specific differences in physiology on $\delta^{18}O$ of body water and tissue O of different genera. The model successfully explained the phenomenon that different animal genera from the same location can have quite different tissue isotope compositions. However, for several reasons, Kohn's model is limited in describing the variation within one species: (1) The model calculates the drinking water amount by subtracting the other input fluxes from total water demand, which accumulates all the errors from other input fluxes into that of drinking water. In addition, the amount of drinking water cannot directly reflect the influence of ambient conditions. (2) The model relates the total water demand only to the weight of animals and a genus-specific water economy index, which cannot reflect the seasonal change of total water demand. (3) The model does not consider lactation, which is a major output flux of O especially in dairy cows, and which requires an equivalent input flux. (4) Finally, $\delta^{18}O$ of feed moisture is assumed to be equilibrated with $\delta^{18}O$ of atmospheric humidity, while the $\delta^{18}O$ in feed moisture in fact will change diurnally and thus grazing time and feeding strategy will also influence $\delta^{18}O$ of ingested feed moisture.

For the reasons given above, we extended the Kohn model (we will refer to it as Munich Kohn model or MK model) as follows: (1) Drinking water intake was estimated directly based on water demand and water provision in the feed. The total water demand thus changed with temperature and relative humidity on a daily basis and the milk production of cows can be considered. (2) The body water turnover was considered by adding the O input fluxes to the body water from the previous day. (3) The different keeping conditions (housing versus pasturing) and feeding strategies were considered. For grazing animals this includes consideration of the convolution of both diurnal rhythms, that of grazing and that of $\delta^{18}O$ in feed. In consequence, only the general principle of creating an animal's mass balance for water and O were taken from the original Kohn model while the details had to be modified.

By knowing $\delta^{18}O$ of body water from the MK model, the $\delta^{18}O$ in hair was predicted and compared to the five-year variation of $\delta^{18}O$ in hair of a domestic suckler cow subject to significantly different seasonal keeping strategies. The MK model will be useful to validate a reported origin and feeding strategy of domestic animals by measuring $\delta^{18}O$ in hair.

Materials and Methods

Keeping and feeding strategy. The sampling was performed at Grünschwaige Experimental Station, Germany (48°23′N, 11°50′E), where a grazing experiment[24] has existed between 1999 and 2012. The animals were kept on an organic farm approved by Naturland e.V., whose regulations also covered the standards of Canadian Council on Animal Care[25]. No other actions than necessary for animal husbandry were carried out. A Limousin suckler cow was selected from a herd of about 10 animals, which was in its second gestation and had a body weight of 637 kg at first hair sampling. The cow suckled a calf in the periods of 09 Dec 2000~22 Nov 2001, 11 Jun 2002~30 Mar 2003, 26 May 2003~14 Jan 2004 and 12 May 2004~07 Jan 2005. The herd was on paddock No. 8, 11, or 13; for paddock properties see Schnyder *et al.*[24]; for an overview of temporal changes in keeping conditions, lactation periods and sampling events see Supplementary Fig. S1).

The animals remained entirely at pasture during grazing seasons and during this time they did not have access to housing and did not receive any supplements except for minerals. During winter, the herd was kept in an open-front free stall (length 55 m, height of the open front 3.75 m; 12 m depth of the stall including the feeding table at the open front) with additional eave and ridge ventilation. A mixture of silage and hay, which came from the same farm, was fed during the stall period. In contrast to fresh grass, silage water after extraction from the silo is close to drinking water because the mown grass equilibrates with the soil water during wilting (−9.1 to −12.9‰, Sun *et al.*[17]). The silage and hay was provided in the morning and remained on the feeding table until the next morning when any remaining orts were removed. The silage was taken to the stall from an open-front, drive-in silo, in which the silage face was exposed to air for about one day before feeding.

The crude protein contents in the feed dry matter were $15.3 \pm 1.4\%$ (grass, $n = 16$) and $12.9 \pm 0.45\%$ (silage + hay, $n = 4$) on average for grazing seasons and stall seasons. In all seasons, the cow had free access to drinking water which was taken from local ground water.

Hair and water sampling and isotope analysis. Sampling carried out during animal weighing on the farm comprised only tissue (hair) that was dead prior to sampling (approved by Technische Universität München). At the beginning and end of the grazing seasons of 2001–2004, hair was collected from the tail switch of the cow (for sampling dates see Supplementary Fig. S1). The hair was cleaned and cut into segments of 1 cm

length (for details, see Auerswald *et al.*[8]). These segments were packed in silver cups (4 to 6 mm) and analyzed by the pyrolysis method in a continuous flow system with an elemental analyzer (EURO EA 3028; Euro Vector, Milan, Italy) interfaced to an IsoPrime isotope ratio mass spectrometer (GV Instruments, Manchester, UK). Each sample was measured against a CO-reference gas calibrated against a secondary isotope standard (benzoic acid, IAEA-601). Stable isotope ratios ($^{18/16}$O) are given in δ notation and expressed in per mil:

$$\delta(‰) = (R_{sample}/R_{standard} - 1) \times 1000‰,$$

where R is the ratio of heavy to light isotopes. Standard is VSMOW (Vienna Standard Mean Ocean Water). For brevity, we only write δ with a subscript to indicate the substance under focus, thus δ_{hair} denotes O isotope composition in hair.

Air vapor (n = 86), precipitation (n = 90), groundwater (n = 87), stem water (n = 162) and soil water at 7 cm (n = 160) was sampled every one or two weeks at Grünschwaige Experimental Station from 2006 to 2012. Stems were not sampled on days when dew or rain was adhering to the grass. Furthermore, days with frozen or snow covered soil were excluded because animals were in the stalls under these conditions. The samples were then stored in a −18 °C freezer until water extraction by cryogenic vacuum distillation (2 h at 80 °C). δ^{18}O in water samples was measured using Cavity Ring-Down Spectroscopy (CRDS, Picarro, USA). Each sample was measured repeatedly (more than four injections) and the values of the last two measurements were averaged (SD for repeated measurement was ±0.1‰). After every 20 to 25 samples, two laboratory water standards, derived from local deionized tap water by evaporation/condensation processes and covering the range of the isotope compositions of the samples, were measured for possible drift correction and normalizing results to the VSMOW scale. The laboratory standards were previously calibrated against V-SMOW, V-GISP and V-SLAP (from IAEA) using the same analytical procedure as used in sample analysis.

Position-time assignment of δ_{hair} segments. To convert the position along the hair to a certain date, the hair growth rate needs to be known. Corresponding sections on hair shafts from subsequent samplings, which had grown at the same time, were localized by statistical isotopic pattern matching[26]. The hair growth rate was then given as length of the newly grown part of the younger hair per time interval between successive sampling dates. Hair growth rates were additionally validated by evaluating the ^{13}C and ^{15}N pattern of replicate hairs in the same way. Hair growth rate was 0.76 mm/d and varied little (slightly lower during stall periods and slightly decreasing with increasing age of the animal; for details, see Auerswald *et al.*[8]). Hair growth rate was then used to interpolate between two subsequent sampling intervals and to assign a growth period to each 1 cm length segment of hair. On average, 1 cm of hair corresponded to a growth period of 13.2 d. Hairs usually comprised more than one year while the sampling interval was 0.5 yr in most cases (Supplementary Fig. S1). Thus, two to three hair segments from different hairs covered the same growth period.

Modelling. *General principle.* The δ^{18}O of body water (δ_{bw}) results from the quantities (M in mole/d) and isotopic compositions (δ in ‰) of O input fluxes (air O uptake, air water vapor into the lungs, chemically bound O in feed, feed moisture and drinking water) and output fluxes (CO_2 production, fecal water, milk water, orally and nasally exhaled respiratory water, O contributing to organic products, sweat water, transcutaneous water vapor, urea and urinary water), which must balance[2]:

$$\begin{aligned}&\left(M_{air} \times d_{air} + M_{vapor} \times d_{vapor} + M_{bO} \times d_{bO} + M_{fw} \times d_{fw} + M_{dw} \times d_{dw}\right)/M_{inputO}\\ &= \left(M_{CO_2} \times d_{CO_2} + M_{fecal} \times d_{fecal} + M_{milk} \times d_{milk} + M_{oral} \times d_{oral}\right.\\ &\quad + M_{nasal} \times d_{nasal}\\ &\quad + M_p \times d_p + M_{sweat} \times d_{sweat} + M_{cutan} \times d_{cutan} + M_{urea}\\ &\quad \left.\times d_{urea} + M_{urinary} \times d_{urinary}\right)/M_{outputO}\end{aligned} \quad (1)$$

The amount of metabolic water results from the chemically bound H in digested feed[2] minus the amount of H required for urea production; its O originates from the chemically bound O in feed and from air O. A description of all variables, their abbreviations and units is given in Supplementary Table S1.

In order to consider the turnover of body water in the MK model, the body water at day i was calculated by adding the O input fluxes to the body water of day i-1:

$$\begin{aligned}&\left(M_{inputO} \times \delta_{inputO} + M_{bw,i-1} \times \delta_{bw,i-1}\right)/\left(M_{inputO} + M_{bw,i-1}\right)\\ &= \left(M_{outputO} \times \delta_{outputO} + M_{bw,i} \times \delta_{bw,i}\right)/\left(M_{outputO} + M_{bw,i}\right)\end{aligned} \quad (2)$$

Fecal water, milk water, sweat water and urinary water are derived from body water without fractionation[2]. Thus, their isotopic composition was replaced by $\delta_{bw,i}$. The output fluxes subject to fractionation (CO_2 production, O in organic products, urea, respiratory and transcutaneous water vapor) resulted from $\delta_{bw,i} + \varepsilon$, where ε denotes the isotopic fractionation between an output flux and body water. Therefore $\delta_{bw,i}$ is solved:

$$\begin{aligned}\delta_{bw,i} = &\left(M_{inputO} \times \delta_{inputO} + M_{bw,i-1} \times \delta_{bw,i-1} - M_{oral} \times \varepsilon_{oral} - M_{nasal} \times \varepsilon_{nasal}\right.\\ &\left. - M_{cutan} \times \varepsilon_{cutan} - M_{CO_2} \times \varepsilon_{CO_2} - M_p \times \varepsilon_p\right)/\left(M_{inputO} + M_{bw,i}\right)\end{aligned} \quad (3)$$

At the same time, the water mass balance of an animal was assumed to be zero,

$$M_{\text{input } H_2O} - M_{\text{output } H_2O} = 0, \quad (4)$$

As well as the oxygen mass balance:

$$M_{\text{input O}} - M_{\text{output O}} = 0, \quad (5)$$

The details of calculation of the MK model are given in Supplementary Tables S2 and S3 together with the sources from which the equations were taken. These equations were selected to cover a wide range of conditions. For example, the equation for respiration was derived for ambient temperatures between −12 °C to +40 °C[27]. In extreme cases not covered by these sources and especially in cases of other domestic ruminants like sheep or goat these equations should be replaced by more appropriate ones.

Input fluxes. Air O uptake and water vapor intake were calculated based on body weight, temperature and relative humidity according to Kohn[2]. The amount of chemically bound O from feed results from the chemical composition of the feed, digestibility and dry matter intake. The dry matter intake was calculated from metabolizable energy, which was determined by milk production, days in gravidity and body weight. The weight was measured at every occasion of movement from stall to pasture and back. Body weight was linearly interpolated between two measurements. In case of high yielding cows a considerable change in weight may occur especially during early stages of lactation due to the melt down of body reserves. The metabolism of body reserves was not considered because O released from the degradation of body lipids and proteins contributes very little to the total O intake. In case that 1 kg d^{-1} of body reserves would be metabolized (not including the export to milk, which is not relevant for the water balance of a cow) and this would contribute only 0.1% to the total O intake, which is irrelevant quantitatively but also isotopically.

Feed moisture was calculated as sum of internal water and adhering water from intercepted rain and soil water from dew rise. The internal water represented grass water in leaf and stem in grazing seasons and silage and hay water in stall seasons. Water contents of fresh grass were obtained from long-term measurements during the growing seasons at Grünschwaige Experimental Station. For the low canopy height of our pasture (compressed height of the sward was controlled to be 7 cm) we estimated that most of the intake comprised leaves (90%) and the remainder (10%) being (pseudo-) stems in grazing seasons. In the Kohn model, drinking water was calculated by subtracting the other input fluxes from total water demand, which did not reflect the seasonal variation of drinking water intake. We used the models described by Cardot *et al.*[28] for the stall period and Sun *et al.*[15] for the grazing period to estimate drinking water intake as a function of ambient conditions (relative humidity, daily average temperature and precipitation), milk production, and soil water storage (during grazing). The Sun model was modified to consider the influence of body weight (an increase of 1 kg body weight causes 0.1 kg increase in drinking water[14]). Sun's model also yielded the amounts of intercepted rain water and soil water from dew rise, which adheres to the grazed grass.

Output fluxes. The estimation of CO_2 production depended on the ingested feed, digestibility, an energy extraction factor, and the amount of O flowing into organic products (milk, growth). Kohn[2] assumed the energy extraction factor to be 0.9 in herbivores, which is not applicable for cows because, in contrast to monogastric herbivores, ruminants lose a considerable amount of energy as methane. Metabolizable energy is thus only 82% of digestible energy[29]. From an isotope point of view, the distinction of fecal, urinary and sweat water appeared unnecessary, given that these body fluids are formed from body water without obvious fractionation[30–32]. Hence these excretions were considered together. Their amount was calculated as the difference between total water intake and all other water losses. The respiratory water was estimated from air flow through the lungs according to Stevens[27] who considered air temperature. We assumed that two thirds of the respiratory water output was from oral expiration and one third was from nasal expiration. The equation by Stevens[27] covers a range in temperature between −12 °C to +40 °C and thus also considers panting, but in our case panting did not occur because the temperature–humidity index was always lower than 78, which is considered the threshold above which a cow starts to pant[33]. Calves suck usually 5–10 kg/d from suckler cows[34]; following Häusler *et al.*[35] we used a linear decrease from birth (10 kg/d) to weaning (5 kg/d) at a rate of 0.02 kg/d^2. The transcutaneous vapor was estimated from ambient conditions using the model built by Maia *et al.*[36]. Further model components came from[37–40].

$\delta^{18}O$ of input and output fluxes. $\delta^{18}O$ of air O utilized in the lungs was set to a typical value of 15.1‰, which is caused by the fractionation during O uptake by the lungs[2]. The $\delta^{18}O$ of air vapor intake into the lungs was estimated from a long-term relation between average daily temperature and $\delta^{18}O$ of vapor determined between 2006 to 2012 at the research site (in total 80 measurements; $\delta_{vapor} = 0.34 \times T_{av} - 21.52$; $R^2 = 0.49$; $p < 0.05$).

In the Kohn model the feed moisture is considered to be equilibrated with air vapor. However, the $\delta^{18}O$ of leaf water in grass changes seasonally and diurnally. To account for this change, MuSICA (Multi-layer Simulator of the Interactions between a vegetation Canopy and the Atmosphere) was parameterized for the research pasture and validated with six years of eddy covariance measurements and with the $\delta^{18}O$ data for soil, stem and leaf water average[41]. MuSICA is a process-based, isotope-enabled model that simulates the exchanges of mass (water, CO_2) and energy in the soil-vegetation-atmosphere continuum as well as the isotopic composition of ecosystem water pools. For details of the MuSICA parameters and validation see Ogée *et al.*[42]. MuSICA can be run in 30-min time steps over multiple years or decades[42,43]. The range of diurnal variation in 1-hr steps is given in Supplementary Fig. S2. We assumed there were four feeding peaks within a day (6:00, 11:00, 15:15 and 21:30)[44] and the feed intake during these four periods to be equal to obtain the mean $\delta^{18}O$ of the ingested leaf water for each individual day. In contrast to leaves, the water in stems is not enriched by transpiration. $\delta^{18}O$ of stem water was set equal to

the long-term (2006 to 2012) monthly isotope measured data of stem water at the research site. $\delta^{18}O$ of precipitation was also used for that of adhering water. The $\delta^{18}O$ of winter feed (silage and hay) water was calculated based on the model built by Sun *et al.*[17] and the assumption that the silage was exposed to air for 24 h. The seasonal change of feed bound $\delta^{18}O$ was not measured. For simplicity it was assumed to be the same as in its main constituent cellulose. $\delta^{18}O$ in cellulose during grazing seasons was estimated from $\delta^{18}O$ of stem water and leaf water during the previous 30 d according to Cernusak *et al.*[45]. In stall seasons it was assumed to be equal to the average growing-season cellulose O. The $\delta^{18}O$ of drinking water was derived from the long-term measurement of ground water (88 measurements), which was almost constant.

The $\delta^{18}O$ of organic products (milk constituents, fetal growth) was determined from the fractionation between body water and protein, which was assumed to be 15‰ according to results obtained for cows[46] and woodrats[23,46]. The $\delta^{18}O$ of other output fluxes was determined from δ_{bw} and the specific fractionation values between output fluxes and body water for a herbivore following Kohn[2].

Hair. δ_{hair} was predicted from $\delta^{18}O$ of body water. O'Gady *et al.*[47] report a fractionation of 16.4‰ while Podlesak *et al.*[23] give a range between 13‰ and 17‰, which is similar to the range found between milk water and milk protein (14 to 16‰[46]). The reason for this wide range is unknown. Hence we used a mean value (15‰)[23] in the first simulations. In a final simulation we treated the body water-keratin fractionation as a fitting parameter to obtain the best value under our conditions.

Input data. The input data of the model contained parameters including weather data, $\delta^{18}O$ of vapor, of precipitation, of stem water, of soil water, and of groundwater, MuSICA output, soil properties, feed properties, and animal properties. The weather data (average daily temperature, minimum temperature, relative humidity, precipitation, vapor pressure) were obtained from the Munich airport meteorological station (about 3 km from the grassland site) operated by the German Weather Service. From $\delta^{18}O$ of water measured in Grünschwaige Experimental Station the long-term biweekly $\delta^{18}O$ of precipitation and of stem water were determined and used as estimates of $\delta^{18}O$ of intercepted water and of stem water; the long-term average $\delta^{18}O$ of ground water was used as the $\delta^{18}O$ of drinking water; long-term daily vapor data were used to evaluate the relationship between temperature and $\delta^{18}O$ of vapor.

The MuSICA model delivered the $\delta^{18}O$ of leaf water on an hourly basis. Soil parameters were taken from Schnyder *et al.*[24] and plant available water was modelled on a daily basis, following these authors. The water contents of silage and hay ($n = 137$) were recorded during the experiment. The digestibility of feed was determined according to the fecal nitrogen method, which proved to be the best method under the experimental conditions[48]. The crude protein needed for this calculation was obtained from the nitrogen content of the grass.

Statistics. Simple linear regressions were used to analyze the relation between two parameters. Paired t-test was used to compare the difference between average $\delta_{hair\_modelled}$ and $\delta_{hair\_measured}$. The root mean squared error (RMSE) was used to quantify mean deviations between prediction and measurement. In order to investigate how much the variation of different O sources and ambient conditions affected the variation of δ_{hair}, individual influxes or ambient conditions were set constant to their long-term mean. The change in variation compared to the full model reflected the influence of this parameter. This approach can only quantify the influence of the variation but it does not reflect how much an individual flux causes the body water to change. This was quantified by calculating isofluxes, which are given by the difference in $\delta^{18}O$ between a flux and that of the body water multiplied by the daily flux rate. The average relative isoflux ($C_{average\_j}$) of flux j is thus given by:

$$C_{average\_j} = M_j \times |\delta_{bw} - \delta_i| / \Sigma\left(M_j \times \left|\delta_{bw} - \delta_j\right|\right) \times 100 \quad (6)$$

where M_j and δ_j are the O amount (mole) and $\delta^{18}O$ of the flux j, respectively. δ_{bw} is the $\delta^{18}O$ of body water at day i.

Significance, if not explicitly stated, always refers to $p < 0.05$. Data are presented as mean values ± standard deviation.

Results

Relations between $\delta_{hair\_measured}$ and ambient moisture sources (vapor, precipitation, soil, plants). δ_{precip} had significant seasonal variation, with monthly averages ranging from −13.2 to −6.6‰ (Fig. 1). It was higher in grazing seasons (−7.2 ± 2.2‰) than in stall seasons (−10.2 ± 2.0‰). $\delta_{hair\_measured}$ (varying from 6.5 to 10.4‰) also was higher in grazing seasons (10.0 ± 1.1‰) than in stall seasons (7.1 ± 1.0‰) yielding about the same difference between seasons as precipitation. For the grazing seasons, averages of $\delta_{hair\_measured}$ and δ_{precip} had a significant linear relationship on a monthly scale ($p < 0.01$, $R^2 = 0.873$, $N = 5$) whereas the relationship was not significant for stall seasons ($p = 0.38$, $R^2 = 0.133$, $N = 7$).

Measured leaf water was significantly enriched compared to precipitation, soil water and stem water because of plant transpiration (Supplementary Fig. S2). The $\delta^{18}O$ of soil water, stem water, leaf water and precipitation were all positively related while the relation between leaf and precipitation was not significant (Supplementary Fig. S2). The R^2 between stem water and soil water was highest ($R^2 = 0.77$) and values were close to the 1:1 line.

Dependence of $\delta_{hair\_measured}$ on temperature, humidity and VPD. In grazing seasons the average monthly temperature (15.1 ± 4.6 °C) was significantly higher than that in stall seasons (3.3 ± 5.4 °C). However, there was no significant difference in monthly relative humidity between grazing seasons (73 ± 12%) and stall seasons (81 ± 13%). Linear relations between temperature (or relative humidity or vapor pressure deficit) and $\delta_{hair\_measured}$ were all significant, but the relations differed between grazing and stall seasons (Fig. 2). During the

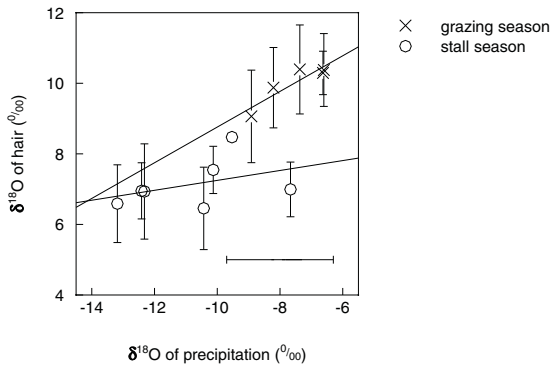

Figure 1. Relationships between monthly averages of measured δ_{precip} and $\delta_{hair\_measured}$. Crosses (grazing season) and circles (stall season) represent the multi-year averages for each specific month when the cow was entirely either in stall or on pasture (90 and 97 hair data and 53 and 27 precipitation data in grazing season and stall season, respectively). The R^2 of the linear regressions are 0.87 ($p < 0.01$) and 0.13 ($p = 0.38$), respectively in grazing and stall seasons, and 0.65 ($p < 0.01$) when taken together. Error bars denote standard deviations. Note that for simplification the horizontal error bar represents only the average of the monthly standard deviations.

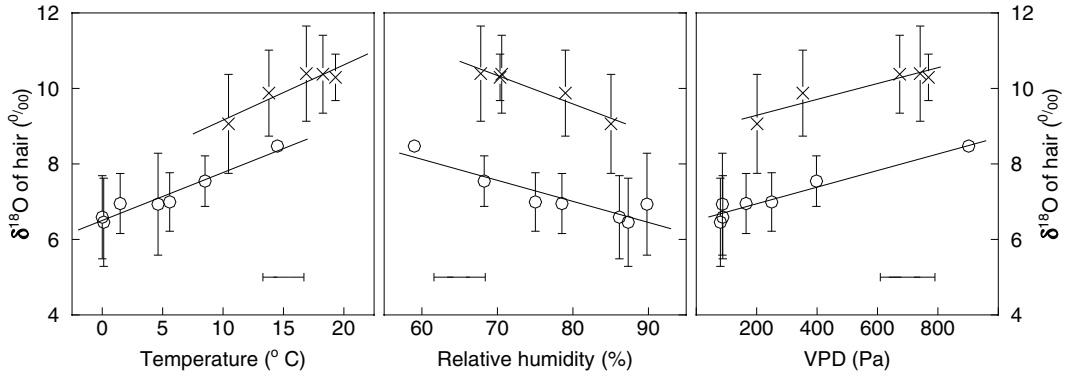

Figure 2. Relationships between monthly averages of $\delta_{hair\_measured}$ and air temperature, relative humidity and vapor pressure deficit (VPD). Crosses (grazing seasons) and circles (stall seasons) represent the averages from 2000 to 2004 for each specific month where the cow was entirely either in stall or on pasture (90 and 97 hair data and 909 and 733 daily ambient conditions data in grazing season and stall season, respectively). The R^2 of a linear regression between temperature (or relative humidity or VPD) and δ_{hair} in grazing and stall seasons was always significant ($p < 0.01$) (for temperature: $R^2 = 0.65$ and $R^2 = 0.70$, respectively; for relative humidity: $R^2 = 0.93$ and $R^2 = 0.82$, respectively; for VPD: $R^2 = 0.87$ and $R^2 = 0.94$, respectively). Error bars denote standard deviations. Note that for simplification the horizontal error bars represent only the average of the monthly standard deviations.

grazing season the hair was more enriched than in the stall season, even when temperature, relative humidity or vapor pressure deficit were identical.

Modelled O input and output fluxes through the cow's body water. On average, the modelled drinking water (2359 mole d^{-1}) and fecal, urinary and sweat water (together 2639 mole d^{-1}) were the highest input and output fluxes, respectively, while the modelled air vapor intake into the lungs (39 mole d^{-1}) and the urea excretion (4 mole d^{-1}) were the lowest fluxes (Fig. 3). Of the input fluxes, the range of drinking water intake was highest, followed by feed moisture. Among the output fluxes, the amount of fecal, urinary and sweat water varied most, followed by transcutaneous vapor, milk water, orally exhaled water and nasally exhaled water, while all other output fluxes had a comparably narrow range.

The main variations of total input and output fluxes were caused by the fluctuations of drinking water intake and fecal, urinary and sweat water (Fig. 4), which were driven by ambient conditions (mainly temperature but also precipitation and soil moisture content). The amount of feed moisture was lower in stall seasons than that in grazing seasons, which was compensated by higher consumption of drinking water in the stall (Fig. 4). There was an exception in the grazing season of 2003: the amount of feed moisture was low because it was an exceptionally dry summer with insufficient grass growth; it was necessary to supplement grazed grass with hay during this grazing season. The contribution of feed moisture to the total input flux was $33.3 \pm 7.5\%$ during grazing seasons and $16.8 \pm 1.6\%$ during stall seasons (Supplementary Table S4). The proportions contributed by drinking water were $54.0 \pm 7.9\%$ in the grazing seasons and $68.1 \pm 2.6\%$, in the stall seasons. Both sources thus contributed the

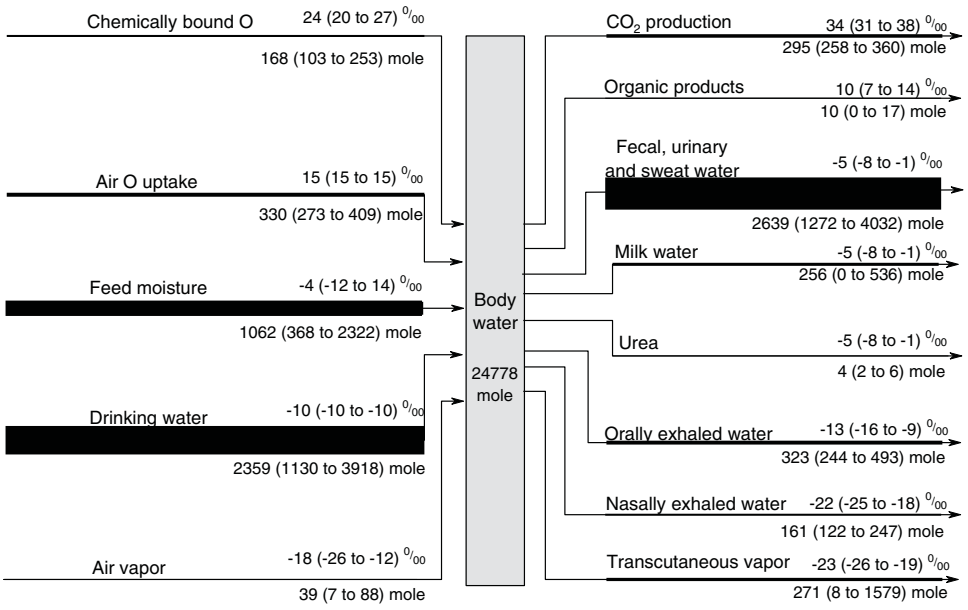

Figure 3. Modelled daily O input and output fluxes through the body water of a suckler cow. Values below and above the lines denote the mean and range (in parentheses) of the flux rates (mole d^{-1}) and $\delta^{18}O$ (‰) for the years 2000 to 2004, respectively. The fluxes are ordered according to $\delta^{18}O$. Line width is proportional to flux rate. The mean and range of $\delta^{18}O$ in body water is identical to fecal, urinary and sweat water.

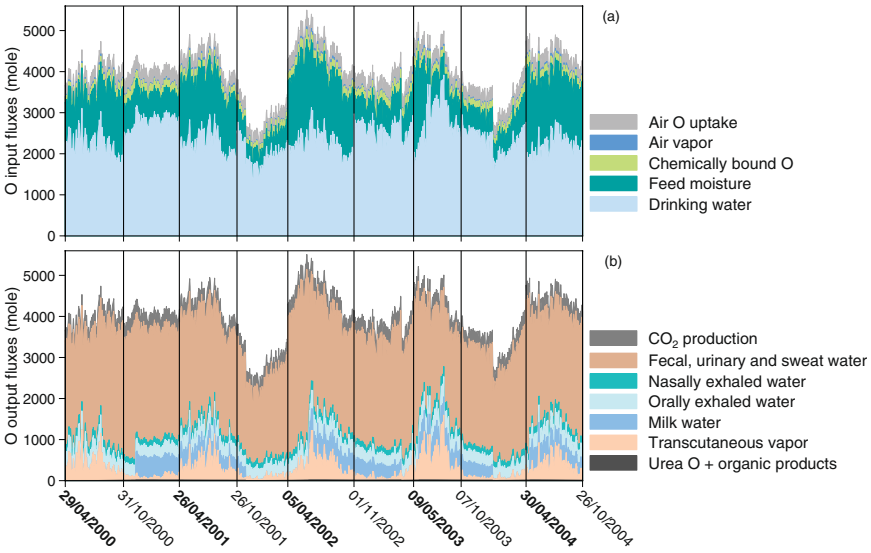

Figure 4. Modelled O amounts of input (**a**) and water output fluxes (**b**) during five years. Vertical lines and time labels show times of diet shift (bold labels indicate start of grazing; normal labels indicate start of stall seasons).

largest share of input fluxes in both seasons. In contrast, air O uptake, air vapor intake into lungs, and chemically bound O of feed contributed relatively little to the O input fluxes (grazing seasons: 7.7 ± 0.5%, 1.1 ± 0.2%, and 3.9 ± 0.5%, respectively; stall seasons: 9.5 ± 1.0%, 0.7 ± 0.2%, and 4.8 ± 0.5%, respectively).

Fecal, urinary and sweat water were the main O output fluxes in our model, contributing 64.3 ± 7.5% during grazing seasons and 70.6 ± 3.4% during stall seasons to the total output flux. CO_2 production, milk water, orally exhaled water, transcutaneous vapor, nasally exhaled water, urea and organic products contributed relatively little to O output fluxes (grazing seasons: 7.1 ± 0.5%, 6.6 ± 4.6%, 8.3 ± 0.8%, 9.4 ± 4.9%, 4.1 ± 0.4%, 0.1 ± 0.0% and 0.2 ± 0.1%, respectively; stall seasons: 8.8 ± 1.0%, 5.6 ± 5.1%, 8.2 ± 1.3%, 2.6 ± 1.8%, 4.0 ± 0.6%, and 0.1 ± 0.0% and 0.3 ± 0.1%, respectively).

Modelled $\delta^{18}O$ of air O uptake was above ambient air O because of the fractionation during O uptake by the lungs. $\delta^{18}O$ of air O uptake and of drinking water were constant (Fig. 5) while all other fluxes varied seasonally in $\delta^{18}O$. The range within other individual output fluxes was about 7‰ while the variation within other individual

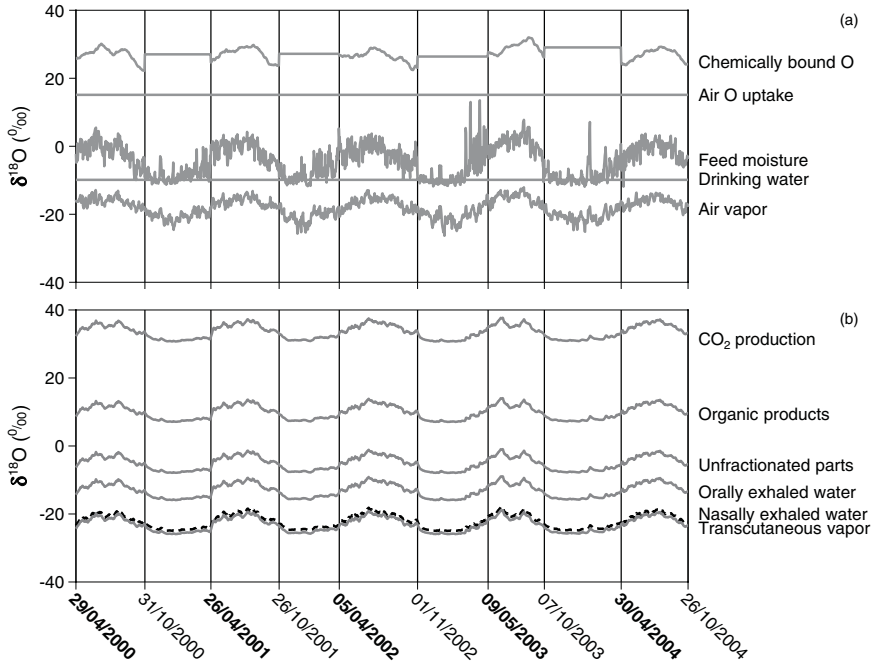

Figure 5. Modelled $\delta^{18}O$ of input (**a**) and output fluxes (**b**) during five years. Vertical lines and time labels show times of diet shift (bold labels indicate start of grazing; normal labels indicate start of stall seasons).

input fluxes was 7‰ or more. Feed moisture exhibited the largest variation (26‰) and, on average, it was more enriched during grazing seasons (-1.2 ± 2.8‰) than during the stall seasons (-8.0 ± 3.5‰). Modelled $\delta^{18}O$ of chemically bound O was the most enriched input flux (grazing seasons: 23.8 ± 1.9‰; for the stall seasons the average over the previous growing season was assumed) (Fig. 5). In warmer months, like July and August, the values were relatively higher than in other months. Modelled $\delta^{18}O$ of air vapor (grazing seasons: -16.2 ± 1.6‰; stall seasons: -20.5 ± 1.9‰) also had significant seasonal variation following temperature.

The isotope compositions of all output fluxes were determined by that of body water and constant fractionations. In consequence, the modelled $\delta^{18}O$ of all output fluxes exhibited the same fluctuations over time even though they differed in absolute values due to different fractionations (Fig. 5). Higher values appeared during warm seasons than during cold seasons and also the fluctuation was larger during grazing seasons (SD: 1.4‰) than during stall seasons (SD: 0.7‰). In decreasing order the average values of $\delta^{18}O$ for CO_2 production, organic products, unfractionated fluxes, orally exhaled water, nasally exhaled water and transcutaneous vapor were 35.1‰, 11.5‰, −3.6‰, −11.6‰, −20.6‰ and −21.6‰ in grazing seasons and 31.4‰, 7.9‰, −7.2‰, −15.2‰, −24.3‰ and −25.2‰ in stall seasons.

Three parameters, namely drinking water, feed internal water and ambient conditions influencing the animal, were the main sources of variation in δ_{hair} (Supplementary Fig. S3), while the other parameters had little influence on the variation. The variation in drinking water intake dampened the fluctuation of hair in whole years (by 0.2‰) and grazing seasons (by 0.4‰). Feed internal water explained more than half of the variation of δ_{hair} within years (1.3‰) and within the grazing seasons (0.9‰); however, it explained only a small part (0.06‰) of the variation in stall seasons (Supplementary Fig. S3). Ambient conditions influencing the animal also caused about half of the variation of δ_{hair} within years (equal to 1.1‰), within grazing seasons (0.5‰) and within stall seasons (0.4‰). Hence modelling showed that almost half (46% within years, 36% within grazing seasons and 52‰ within stall seasons; Supplementary Fig. S3) of the seasonal variation in body water resulted from the animal itself and not from the feed. This may be easily overlooked due to the similarity in the seasonal variation of feed and body water, both of which are exposed to and transpire in the same environment.

A different picture was apparent from the calculation of isofluxes (Table 1). Expired CO_2, drinking water, air O uptake and chemically bound O had the largest influence on body water because of their large isotopic contrast to body water (Fig. 3). Except for expired CO_2 and chemically bound O, these fluxes did not vary in $\delta^{18}O$ and thus dampen the isotopic variation of body water. As a result of this dampening effect, body water of the animal varied less than feed moisture (Fig. 5) although the combined and synchronous effects of feed moisture and ambient conditions influencing the animal would suggest that body water varies more than feed moisture. The resulting synchrony of feed moisture and body water then caused the isoflux of feed moisture to become small (Table 1) despite its pronounced seasonal variation in amount and $\delta^{18}O$.

Relationship between ambient conditions (humidity and temperature) and modelled input and output fluxes of O. The modelled input proportions of air O uptake, chemically bound O in feed, and feed moisture increased with increasing relative humidity in grazing seasons (Fig. 6, upper panels). This was

Flux	Whole year	Grazing seasons	Stall seasons
CO_2 production	27	24	32
Drinking water	22	26	16
Air O uptake	14	11	18
Chemically bound O	10	8	13
Transcutaneous vapor	9	14	4
Nasally exhaled water	7	5	7
Orally exhaled water	6	5	5
Feed moisture	5	5	3
Air vapor	1	1	1
Organic products	1	1	1
Unfractionated output fluxes	0	0	0

Table 1. Average relative isoflux contribution (%) to the change of $\delta^{18}O$ in body water by different fluxes. The relative isoflux contribution depends on the isotopic spacing between the flux and the body water and the amount of the flux.

compensated by a decreasing proportion of drinking water with increasing relative humidity. In the stall seasons, humidity had no influence on the amount of all input fluxes.

Modelled $\delta^{18}O$ of air O uptake and drinking water was independent of relative humidity (Fig. 6, lower panels) while $\delta^{18}O$ of air vapor and feed moisture decreased with relative humidity in both seasons. The $\delta^{18}O$ of chemically bound O decreased only during the grazing seasons with increasing relative humidity, while there was no influence of actual humidity during stall seasons on bound O because it originated from the previous growing season.

Modelled proportions of air O uptake, chemically bound O and feed moisture contributing to total water intake decreased in both seasons when the temperature increased (Fig. 7, upper panels), while there was an increasing trend for air vapor and drinking water with increasing temperature. The relations for air O uptake, vapor and chemically bound O almost overlapped in different seasons, but were pronouncedly separated for feed moisture and drinking water.

The $\delta^{18}O$ of air O uptake and drinking water did not change with temperature (Fig. 7), while the modelled $\delta^{18}O$ of air vapor was fully explained by temperature from which it was calculated. The modelled $\delta^{18}O$ of chemically bound O in feed was not influenced by temperature in stall seasons but it increased with temperature in grazing seasons, although chemically bound O in feed was the result of growth during preceding days. This relation thus resulted from the higher probability of a warm day following warm days and a cold day following cold days. Modelled $\delta^{18}O$ of feed moisture increased significantly more in stall seasons than in grazing seasons when temperature increased.

During stall seasons, there were two groups in the proportions of O input fluxes (especially pronounced for drinking water proportion in Figs 6 and 7). This separation was caused by the influence of weaning, which always happened during stall seasons (compare Supplementary Fig. S1). Drinking water demand then suddenly decreased because milk production terminated. During grazing seasons these two groups were not obvious because the cow suckled a calf most of the time (compare Supplementary Fig. S1) and the amount of milk gradually decreased with increasing age of the calf.

Hair measurement and modelling. Modelled values were similar to measured values (Fig. 8). The RMSE between $\delta_{hair\_measured}$ and $\delta_{hair\_modelled}$ was 1.4‰ and $\delta_{hair\_measured}$ was not significantly different from $\delta_{hair\_modelled}$ ($p > 0.05$; paired t test). The model estimated the seasonal variation well: the $\delta_{hair\_measured}$ and $\delta_{hair\_modelled}$ almost simultaneously reached the minima in each stall season or the maxima in each grazing season and the modelled minima and maxima were close to the measured values.

Discussion

A mechanistic model for predicting $\delta^{18}O$ in body water turned out to be of high complexity despite the well documented, simple linear relationships between $\delta^{18}O$ in body tissues and rain[9]. The mechanistic modelling required so many parameters that a practical use, e.g. for authenticity testing, is hardly conceivable. The value of such a model is threefold. It compiles our current understanding of the influences on body water $\delta^{18}O$. It allows understanding how the simple relation with $\delta^{18}O$ in rain evolves (see discussion below). And finally, it allows deriving quantitative hypotheses (e.g. on the influences of body size or milk yield or soil) that then can be examined in controlled experiments for identification of gaps in our process understanding. The influences of such boundary conditions are so manifold and interacting that a sound hypothesis can hardly be created without such a model. The model further allows judging, which parameters must be measured or controlled in such an experiment for obtaining reliable results and for describing their range of validity. For instance: one of the most important single number in our modelling turned out to be the plant available water capacity of the soil, a parameter which is hardly ever measured or reported in animal studies. It entered our calculations in several places: (i) it influences water stress of the plant and thus $\delta^{18}O$ in leaf water and chemically bound $\delta^{18}O$. (ii) It influences the mixing of rain

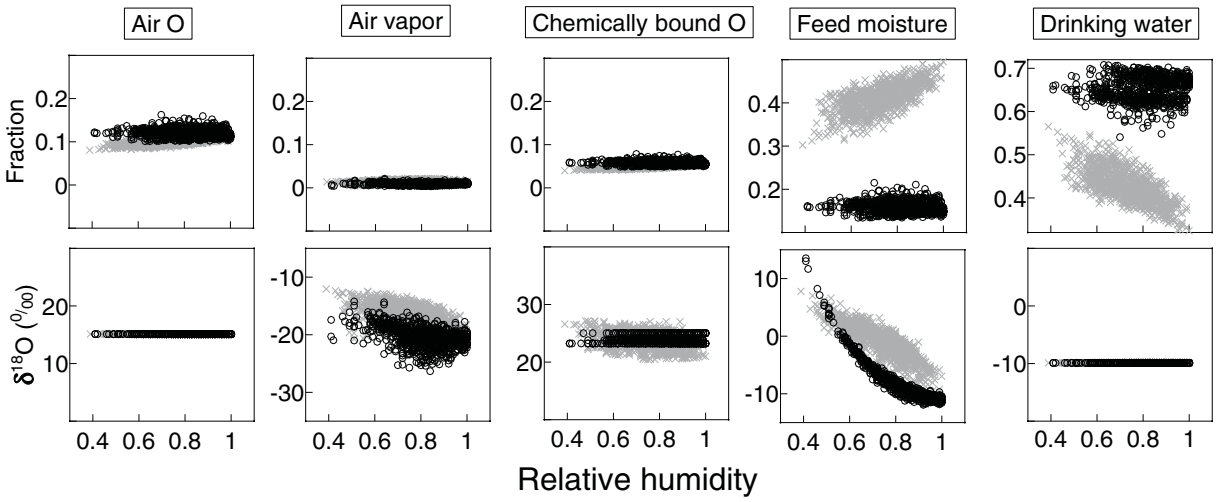

Figure 6. Relationships between relative humidity and modelled fractions of total O input fluxes (upper row) and modelled $\delta^{18}O$ (lower row). Grey crosses and black circles represent grazing and stall seasons, respectively. Note that scaling of the y axes within a row is identical (0.4 for the upper row and 30‰ for the lower row) but the lower boundaries differ between panels.

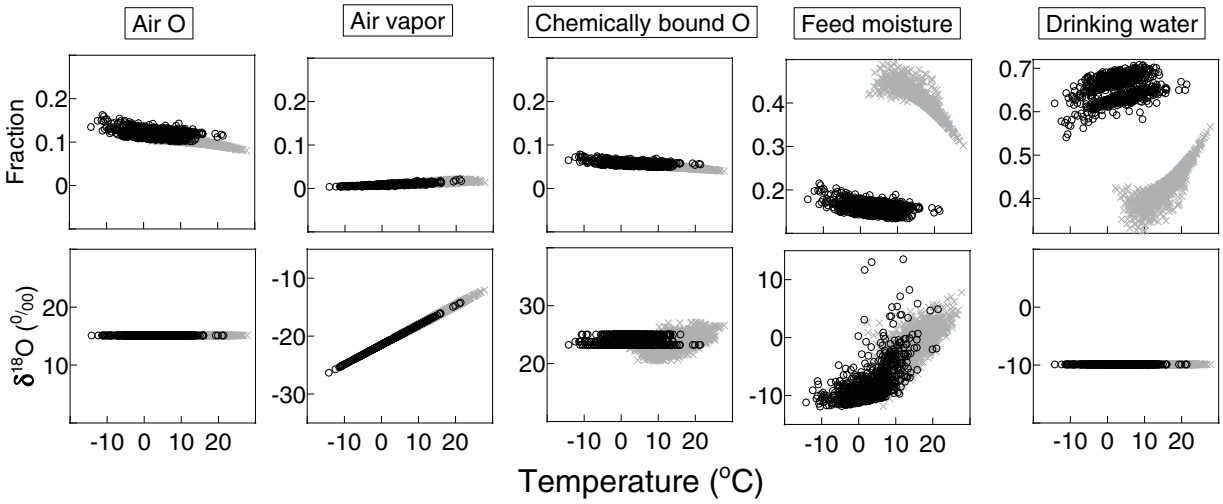

Figure 7. Relationships between temperature and the modelled fractions of total O input fluxes (upper row) and modelled $\delta^{18}O$ (lower row). Grey crosses and black circles represent grazing and stall seasons, respectively. Note that scaling of the y axes within a row is identical (0.4 for the upper row and 30‰ for the lower row) but the lower boundaries differ between panels.

water and thus stem water. (iii) It influences the amount of water adhering to the leaves from dewrise. (iv) And thus, it also influences the drinking water demand of the animals[15].

A major disadvantage of such a complex model requiring many parameters is that it provides ample opportunity for parameter adjustment to improve the fit between prediction and measurement. We took great care not to adjust parameters but to use them as published or measured. E.g., we use the plant available water capacity as published (Schnyder *et al.*[24]) without optimizing it within its range of uncertainty. Only the body water-keratin shift was optimized in a final step (see discussion below).

A second major disadvantage of such a complex model is that every parameter unavoidably carries some error; these are then combined in the model and interact. For instance, we used rainfall data from a station of the German Weather Service in 3 km distance. It is well known that rainfall can vary by a factor of two within a distance of only 1 km[49,50] although long-term rainfall should be identical within this distance. Our model offers the advantage of sensitivity testing to find out, which accuracies are needed for the individual parameters.

The measured δ_{hair} correlated with δ_{precip} during the grazing season, which seems to be in line with the finding of Ehleringer *et al.*[9] that δ_{hair} in humans correlates with $\delta^{18}O$ in tap water on a regional scale, where tap water again reflects the regional variation in δ_{precip}. Such a direct link, however, is true only to a small degree for cows, because δ_{leaf}, which contributed most of the feed moisture during the grazing season, did not correlate with δ_{precip}

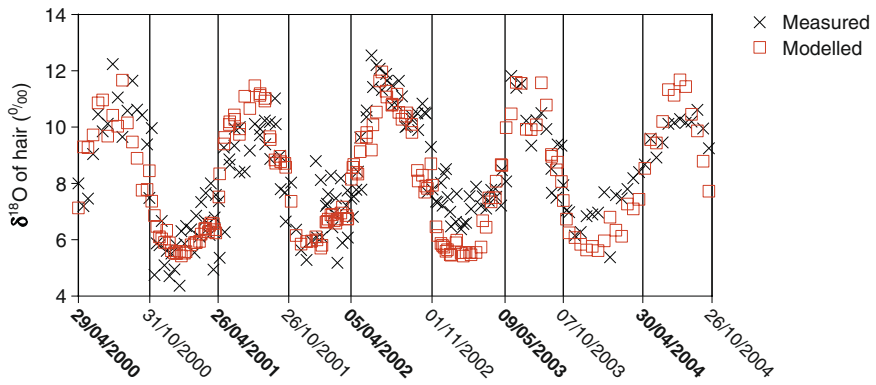

Figure 8. Measured and modelled $\delta^{18}O$ of hair during five years. Black crosses and red circles represent measured and modelled values, respectively. Vertical lines and time labels show starting times of diet shift (bold labels indicate start of grazing; normal labels indicate start of stall seasons). Body water-keratin fractionation was obtained by fitting (14‰).

(Supplementary Fig. S2). Only stem water and precipitation intercepted by the grass carry the isotopic information of precipitation but both contribute little to total water intake. Hence, the close correlation between δ_{precip} and δ_{hair} must originate from indirect relations. The δ_{precip} is closely linked with atmospheric humidity and temperature[51]. Simultaneously, about half of the seasonal variation of δ_{hair} was generated by the animal itself, which in turn was influenced by its ambient conditions (including temperature, air humidity); this created the apparently close relation between δ_{hair} and δ_{precip}. Similarly, the seasonal variation found previously for hair, milk and tooth[2,7,52,53], and which apparently reflected the seasonal variation in feed moisture, resulted only partly from this variation, while the other part was caused by the animal. The detailed and mechanistic modelling of body water and δ_{hair} thus provided insights that easily could be overlooked in regression analyses due to the close correlations with some environmental water sources. An additional example of the seasonal variation caused by the animal itself under constant feeding conditions (total mixed ration and tap water) is given for milk data in Fig. S4 (right panel) in the Appendix.

The large contribution of the animal's ambient conditions to the variation of $\delta^{18}O$ in body tissues is advantageous when $\delta^{18}O$ is used as an indicator of geographic origin because $\delta^{18}O$ in body tissues is then less adulterated by the type of moisture uptake (e.g., the time of grazing or the moisture content of the feed). For the same reason, it is disadvantageous when $\delta^{18}O$ in animal products is used to serve as an indicator of the production system (e.g. to distinguish between milk produced from fresh grass or silage) unless there is additional information available for the ambient conditions.

Lactation has a pronounced influence on drinking water uptake and this has been demonstrated in many previous studies[11,12,14,28]. This influence became especially visible during the stall periods. Drinking water had the lowest $\delta^{18}O$ among all water sources. Nevertheless, this pronounced variation in drinking water uptake caused by lactation was not evident in measured or modelled δ_{hair}. The reason is that lactation also increases feed intake and thus the intake of enriched water during grazing seasons. Both of these lactation-induced changes compensate each other and thereby remove the influence of lactation. Similarly, during stall seasons, no net influence of lactation can result from an increased intake of drinking water because silage water and drinking water are similar in $\delta^{18}O$. The range of milk production of our suckler cow was narrow (10 kg d^{-1}) while a much larger range can be found in dairy cows. Also for this much wider range the influence of lactation on $\delta^{18}O$ in body water is negligible (for an example see Fig. S4 in the Appendix).

Ehleringer *et al.*[9] found that human hair O was enriched by about 22.7‰ compared to O in tap water, while in the case of our study the enrichment was about 3‰ less, although cows incorporate high proportions of highly enriched leaf water with their diet, in contrast to humans. Although Ehleringer *et al.*[9] did not report the average $\delta^{18}O$ in the diet water; it is highly likely that the human diet, prepared mainly using tap water, is less enriched than the water of a cow diet, which consists mainly of fresh leaves. This discrepancy (higher values in humans despite lower values in diet) corroborates a major finding of our study, namely that a large part of the variation in body water results from the animal itself (e.g. transpiration, respiration and water demand). Water losses that cause enrichment of the body water average 23% of the water intake by humans[31], while these losses comprised only 18% of the water intake of our cow. Thus, enrichment by respiration and transpiration can exert a larger influence in humans than in our cow.

The modelled $\delta^{18}O$ of body water varied from −8 to −1‰, which was very close to the values found by Boner and Förstel[4] in Germany (−7 to −1‰), and also the similarity of measured and modelled $\delta^{18}O$ in hair suggests that the mechanistic MK model captures the most important influences. Major uncertainties are likely to be associated with (1) the estimation of $\delta^{18}O$ in feed moisture and in (2) the estimation of δ_{hair} from the $\delta^{18}O$ of body water:

(1) Uncertainty from estimation of $\delta^{18}O$ in feed moisture: During summer there is a major uncertainty in $\delta^{18}O$ of feed because of the unknown diurnal proportions of feed intake, likely to be affected by environmental variables (day length, temperature, rainfall). Based on MuSICA modelling, the diurnal range of $\delta^{18}O$ of leaf water was about 7‰ on average (Supplementary Fig. S5), which agrees with other findings[54,55]. Night-dominated grazing

during a hot period may thus lead to a lower than average $\delta^{18}O$ of ingested leaf water, while daylight grazing on a cool day may provide feed water above average $\delta^{18}O$. Secondly, we assumed a leaf to shoot ratio of feed of 0.9 according to our visual observations, whereas Kohn[2] recommended a ratio of 0.5 for herbivores, and Durham[56] reported a range from 0.56 to 0.84 for the Texas Coastal Prairie. Changing the leaf to shoot ratio in a sensitivity test showed that the mean offset between measurement and prediction would disappear for a leaf to shoot ratio of 0.3. Such low ratios are very unlikely in a canopy of 7 cm compressed sward height and do not comply with our visual observations; however, we are not aware of estimates that would describe the variation under a wide range of conditions.

During winter time, our knowledge and prediction abilities of feed moisture and chemically bound O are even more limited. We had assumed constant values for chemically bound O, while variations are highly likely because silage originates from different fields, from different days and from different harvesting conditions (e.g. time during day). However, it is not possible to predict which portions of silage from a silo are fed on a specific day or the properties these particular portions. Thus, during the stall period the MK model mainly reflected the variation created by the animal but not the variation originating from feed.

(2) Uncertainties of δ_{hair} estimation from the $\delta^{18}O$ of body water: A mechanistic model for δ_{hair} was developed by Ehleringer *et al.*, Bowen *et al.* and O'Grady *et al.*[9,47,57] that shares the general principle of calculating body water from influxes and outfluxes, but it uses constants to describe these fluxes. The model assumes that $\delta^{18}O$ in hair is derived from isotopic exchange with gut water during hydrolysis of dietary protein; gut water, in turn, results from the mixture of food water, drinking water and body water. This model was applied in humans, nonhuman primates, and woodrats. Cows are different to these species in having a four-compartment stomach involved in water absorption and remixing. Absorption and remixing make the prediction of gut water more complicated than in monogastric animals. For example, part of the drinking water may directly reach the omasum by bypass flow via the esophageal groove without mixing with the ruminal water, and some of the saliva can already be absorbed by the rumen[58]. The fraction of bypass flow and its drivers are unknown. For simplicity, a fractionation between body water and keratin derived from the average value in rodents (15‰)[23] was used in our model. The best-fitted value was 14‰, which was close to the aforementioned estimate; however, the mechanisms underlying the fractionation between body water and hair should be investigated further for a range of species.

Recently, identification of food authenticity and geographical origin has become a crucial issue requested both by consumers and authorities because of the frequent global exchange of food. The European Union's general food law (Regulation EC No. 178/2002) has made traceability compulsory for all food and feed businesses since 2005[59]. Multi-element stable-isotope ratio (SIR) analysis has been proved to be practical for this purpose[60]. However, the mechanisms of isotope flow in animals are still not fully understood, especially for $\delta^{18}O$. The application of $\delta^{18}O$ is mainly based on the fundamental fact that $\delta^{18}O$ in wild animal tissue is usually linearly related to $\delta^{18}O$ in annual precipitation, which can be used to detect the geographical origin of animals along precipitation gradients[61]. Application of the MK model showed that this simple relation is the result of the interaction of many processes, and most of these can be manipulated in domestic animals. The $\delta^{18}O$ in domestic animal tissues thus carries the convoluted information of geographic origin and animal husbandry and the MK model may be used for disentangling these influences (for an example of the application and validation of the MK model for milk see Fig. S4 in the Appendix).

There are two parts in the MK model: the estimation of $\delta^{18}O$ of body water and the subsequent estimation of δ_{hair}. The estimation of body water may be useful to identify if there is any fraud in the claimed origin of milk or meat by comparing the measured and modelled water O in them. However, it is indispensable to account for the seasonal variation. The seasonal $\delta^{18}O$ variation of body water was 8‰ in our case. Chesson[62] reported a regression between $\delta^{18}O$ in milk and rain ($\delta_{milk} = 0.86 \times \delta_{precip} + 1.1$), which implies that the regional difference in precipitation must be larger than 9‰ to override the seasonal variation when the time of production is not known.

The second part of the MK model, the estimation δ_{hair}, also has some potential applications. Since the beginning of the Neolithic age about 10,000 years ago, humans have tried to influence the life cycle of domestic animals[7]. The isotopic study of the animal remains (such as hair) may shed some light on animal husbandry. The large number of variables influencing body water and hair, however, calls for a cautious interpretation, especially when ambient conditions are not known in detail.

In our case the MK model was applied in a temperate region where panting did not occur. Panting in heat stress conditions increases the orally exhaled water and thus causes an additional enrichment of the body water but other changes will happen simultaneously (increased transcutaneous vapor, increased drinking water uptake). The MK model offers the advantage to consider all changes in animal physiology simultaneously that are induced with increasing temperature (for an example see Fig. S6 in the Appendix, which shows that increasing transpiration, sweating and panting increases drinking water uptake and thus decreases the contribution of metabolic water to total water intake; the model results fit well to the data by Khelil-Arfa *et al.*[63], who quantified metabolic water to contribute about 5% under thermoneutral conditions (15 °C) and 4% under high-temperature (28 °C) conditions). A much larger effect of high temperatures as found in sub-tropical and tropical latitudes can be expected, however, from the differences in meteoric water, the difference in the diurnal adaptation of feeding and the differences in plant species composition. Under high temperature conditions animals will preferably graze at night[64] when leaf water enrichment in minimal. Plant species composition changes from species with C_3 photosynthesis to species with C_4 photosynthesis, which have a considerably higher enrichment of $\delta^{18}O$ in leaf water[65] due to differences in water use efficiency and which may even exploit different sources of water[66].

Conclusions

The variation of δ_{hair} of a domestic cow results from the interplay of environment, animal physiology and feeding strategy. Temperature and relative humidity were significantly related to measured and modelled δ_{hair} in summer and winter seasons. Temperature and relative humidity not only influenced the feed (e.g. feed internal water

composition) but also the animal itself, (e.g. drinking water intake). Modelling showed that almost half of the seasonal variation in body water resulted from the animal itself. This may be easily overlooked due to the similarity in the seasonal variation of feed and body water, both of which are exposed to and transpire in the same environment.

The mechanistic MK model explained well the variation between seasons and within seasons, although strong indications existed that the influences of animal behavior and animal physiology are still insufficiently understood for predicting $\delta^{18}O$ in animal tissues. Nevertheless, the MK model allows accounting for animal husbandry and feeding strategy in domestic animals. This will foster our understanding of $\delta^{18}O$ in animal products; it will allow identifying those management strategies for which $\delta^{18}O$ in animal products can serve as a reliable proxy. Further tests of this model under different climatic and husbandry conditions in different regions are necessary for a wider application.

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Author Contributions

G.C. and K.A. set up the model, G.C. analyzed the data and wrote the first draft. K.A. put forward the idea of the paper and revised the manuscript. H.S. reviewed the manuscript.

Additional Information

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Supplementary Information

Model explanation of the seasonal variation of $\delta^{18}O$ in cow (*Bos taurus*) hair under temperate conditions

Guo Chen, Hans Schnyder, Karl Auerswald

Table S1: Parameters, abbreviations and units used in this paper; part 1: fluxes.

Flux	abbreviation	unit
Intake of water adhering to feed	M_{adhere}	mole/d
O intake from air	M_{air}	mole/d
Intake of bound H in digested feed	M_{bH}	mole/d
Intake of bound O in digested feed	M_{bO}	mole/d
O in carbon dioxide flux	M_{CO2}	mole/d
Transcutaneous vapor flux	M_{cutan}	mole/d
Drinking water intake on dry day (when $(P_{i-1} + P_i)<0.02$ mm)	M_{dry}	mole/d
Drinking water intake	M_{dw}	mole/d
Fecal water loss	M_{fecal}	mole/d
Nasally exhaled water loss	M_{nasal}	mole/d
Orally exhaled water loss	M_{oral}	mole/d
O in organic products	M_p	mole/d
Exhaled water by respiration	M_{resp}	mole/d
Intake of feed internal water	M_{inner}	mole/d
Total O input flux	M_{inputO}	mole/d
Total water input flux	M_{input_H2O}	mole/d
Total O output flux	$M_{outputO}$	mole/d
Total water output flux	M_{output_H2O}	mole/d
Milk water	M_{milk}	mole/d
Sweat water loss	M_{sweat}	mole/d
Feed moisture uptake	M_{fw}	mole/d
Urinary water loss	$M_{urinary}$	mole/d
O flux with urea	M_{urea}	mole/d
Vapor O uptake by breathing	M_{vapor}	mole/d
Drinking water intake on wet day (when $(P_{i-1}+P_i)>2$mm)	M_{wet}	mole/d

Table S1, continued: Parameters, abbreviations and units used in this paper; part 2: Isotope compositions.

Water or oxygen source	abbreviation	unit
Winter feed before exposure to air	δ_0	‰
Water adhering to feed	δ_{adhere}	‰
O utilized in lungs	δ_{air}	‰
Body water	δ_{bw}	‰
Cellulose	δ_c	‰
Transcutaneous vapor	δ_{cutan}	‰
Bound O in feed	δ_{bO}	‰
Drinking water	δ_{dw}	‰
Feed moisture	δ_{fw}	‰
Hair	δ_{hair}	‰
Measured hair	$\delta_{hair\_measured}$	‰
Modelled hair	$\delta_{hair\_modelled}$	‰
Total O input flux	δ_{inputO}	‰
Leaf water	δ_{leaf}	‰
Nasally exhaled water	δ_{nasal}	‰
Orally exhaled water	δ_{oral}	‰
Total O output flux	$\delta_{outputO}$	‰
O in organic products	δ_p	‰
Precipitation	δ_{precip}	‰
Winter feed moisture in equilibrium with air	δ_{ss}	‰
Stem water	δ_{stem}	‰
O flux with urea	δ_{urea}	‰
Vapor in free air	δ_{vapor}	‰

Table S1, continued: Parameters, abbreviations and units used in this paper; part 3: Oxygen fractionations.

Fractionation	abbreviation	unit
between CO_2 and body water	ε_{CO2}	‰
between transcutaneous vapor and body water	ε_{cutan}	‰
between vapor and water in equilibrium	ε_{eq}	‰
Kinetic fractionation	ε_k	‰
between nasally exhaled water and body water	ε_{nasal}	‰
between oral exhaled water and body water	ε_{oral}	‰
between organic products and body water (15 ‰)	ε_p	‰
between carbonyl oxygen and water	ε_{wc}	‰

Table S1, continued: Parameters, abbreviations and units used in this paper; part 4: Other parameters.

Parameter	abbreviation	unit
Air flow through the lungs	A	L/d
Oxygen content in air (21 %)	C_{air}	%
Average relative contribution of flux j to the change of $\delta^{18}O$ in body water	$C_{average\_j}$	%
Carbohydrate content	C_c	%
Fat content	C_f	%
O conversion factor (0.00216 mole/KJ)	C_o	mole/KJ
Crude protein content	C_p	%
Digestibility	D	
Days in gravidity	d_g	d
Days in milk	d_m	d
Energy extraction efficiency	E_{ex}	
Energy used for heat production	E_H	KJ/d
Metabolizable energy	E_{met}	KJ/d
Energy used for mass production	E_P	KJ/d
Unit conversion factor L → mole (55.56 mole/L)	f_{mL}	mole/L
Unit conversion factor m^3 →mole (3 mole/m^3 vapor at body temperature)	f_{mcm}	mole/m^3
Unit conversion factor d →min (1440 min/d)	f_{mind}	min/d
Unit conversion factor d →s (86400 s/d)	f_{sd}	s/d
Unit conversion factor kJ →Ws (1000 Ws/kJ)	f_{WskJ}	Ws/kJ
Relative humidity	H	
Body weight	m_{animal}	kg
Dry mass intake of feed	m_{dry}	kg/d
Milk production	m_{milk}	kg/d
Oxygen extraction from air (0.2)	O_{ex}	
Proportion of oxygen atoms exchanging with medium water during cellulose synthesis	P_{ex}	
Precipitation at day i	P_i	mm/d
Relative plant available water	P_{rel}	mm/mm
Proportion of unenriched (source) water in tissue where cellulose synthesis is occurring	P_x	
Leaf to shoot ratio of feed	R	kg/kg
Animal surface area	S	m^2
Half-life of silage water	$t_{0.5}$	h
Exposure time of winter feed	$t_{exposed}$	h
Average daily temperature	T_{av}	°C
Minimum daily temperature	T_{min}	°C
Molar gas volume (25.5 L/mole at 38 °C)	V_m	L/mole
Water content of feed fresh matter	W_C	g/g

Table S2: Calculation methods for parameters of input fluxes. The equations were adjusted to common units and simplified where possible compared to original equations of the reported sources.

Parameter	Function	Source
A	$E_H \times C_o \times V_m / O_{ex} / C_{air} \times 100 = E_H \times C_o \times 607$	Kohn[2]
E_H	$(5.6 \times m_{animal}^{0.75} + 1.6 \times 10^{-5} \times d_g^3 + 22 \times m_{milk}) \times f_{sd} / f_{WskJ} = 484 \times m_{animal}^{0.75} + 1.4 \times 10^{-3} \times d_g^3 + 1900 \times m_{milk}$	DIN[37]
E_P	$2 \times (1.4 \times 10^{-3} \times d_g^3 + 1900 \times m_{milk})$	DIN[37]
E_{met}	$E_H + E_P$	Robbins[29]
m_{dry}	$E_{met} / (170 \times C_c + 400 \times C_f + 200 \times C_p) / D / E_{ex}$	Robbins[29]
M_{air}	$2 \times C_o \times E_H$	Kohn[2]
M_{bH}	$2 \times D \times E_{ex} \times m_{dry} \times (0.31 \times C_c + 0.6 \times C_f + 0.11 \times C_P)$	Kohn[2]
M_{bO}	$2 \times D \times E_{ex} \times m_{dry} \times (0.15 \times C_c + 0.02 \times C_f + 0.03 \times C_P)$	Kohn[2]
M_{fw}	$m_{dry} \times f_{mL} \times W_C / (1 - W_C) + M_{adhere}$	This study
M_{dw}	Grazing: (1) If $(P_{i-1} + P_i)<0.02$: $M_{dry} = (0.0011 \times T_{av}^3 + 8.8 + (-0.22 \times H + 13.3 - 0.0011 \times T_{av}^3) \times (P_{rel})^4 + (m_{animal} - 411) \times 0.1) \times f_{mL}$ =$0.061 \times T_{av}^3 - 1794.6 + (-12.22 \times H + 738.9 - 0.061 \times T_{av}^3) \times (P_{rel})^4 + m_{animal} \times 5.6$ (2) If $(P_{i-1} + P_i)>2$: $M_{wet} = (0.0013 \times T_{av}^3 + 4.4 + (-0.22 \times H + 17.7 - 0.0013 \times T_{av}^3) \times (P_{rel})^4 + (m_{animal} - 411) \times 0.1) \times f_{mL}$ =$0.072 \times T_{av}^3 - 2039.1 + (-12.22 \times H + 983.4 - 0.072 \times T_{av}^3) \times (P_{rel})^4 + m_{animal} \times 5.6$ (3) If $0.02<(P_{i-1} + P_i)<2$: $M_{dw} = (M_{wet} + M_{dry}) / 2$ Stall: $(1.53 \times m_{dry} + 1.33 \times m_{milk} + 89 \times (1 - W_C) + 0.57 \times T_{min} - 0.3 \times P_i - 25.65) \times f_{mL}$ = $85 \times m_{dry} + 74 \times m_{milk} - 4953 \times W_C + 32 \times T_{min} - 17 \times P_i + 3525$	Cardot et al[28]; Sun et al.[15]
$M_{input\ H2O}$	$M_{dw} + M_{fw} + M_{vapor} + M_{bH} / 2 - 2 \times M_{urea}$	This study
M_{inputO}	$M_{dw} + M_{fw} + M_{vapor} + M_{bO} + M_{air}$	Kohn[2]
M_{vapor}	$10^{(0.686+0.027Tav)} \times H \times A / 760 / V_m$	Kohn[2]
δ_{vapor}	$0.34 \times T_{av} - 21.52$	This study
δ_{leaf}	MuSiCA modelling	Ogee et al.[42]
δ_{ss}	$(\delta_{vapor} + \varepsilon_{eq} / H + (1 - H) / H \times \varepsilon_k) / (1 + \varepsilon_k / 1000 - 1 / H \times (\varepsilon_k + \varepsilon_{eq}) / 1000)$	Wen[38] Helliker et al.[39]
δ_{fw}	Grazing: $((\delta_{leaf} \times R + \delta_{stem} \times (1 - R)) \times M_{inner} + \delta_{adhere} \times M_{adhere}) / M_{fw}$ Stall: $(\exp(-\ln(2) t_{exposed} / t_{0.5}))(\delta_0 - \delta_{ss}) + \delta_{ss}$	This study Sun et al.[17]
δ_c	$(\delta_{leaf} - \delta_{stem})(1 - P_x \times P_{ex}) + \delta_{stem} + \varepsilon_{wc}$ = $0.58\ \delta_{leaf} + 0.42\ \delta_{stem} + 27$	Cernusak et al.[45]
δ_{inputO}	$(M_{air} \times \delta_{air} + M_{bO} \times \delta_c + (M_{inner} + M_{adhere}) \times \delta_{fw} + M_{dw} \times \delta_{dw} + M_{vapor} \times \delta_{vapor}) / M_{inputO}$	This study

Table S3: Calculation methods for parameters of output fluxes. The equations were adjusted to common units and simplified where possible compared to original equations of the reported sources.

Parameter	Function	Source
M_{CO2}	$M_{outputO} - M_{output\ H2O} - M_{urea} - M_p$	This study
M_{cutan}	$85.18 \times e^{(Tav-24.92)/7.96} \times f_{sd} / (2500.7879 - 2.3737 \times T_{av}) \times S / 18$ $= 408864 \times e^{(Tav-24.92)/7.96} / (2500.7879 - 2.3737 \times T_{av}) \times S$	Maia et al.[36]
$M_{fecal}+M_{urinary}+M_{sweat}$	$M_{output\ H2O} - M_{milk} - M_{cutan} - M_{oral} - M_{nasal}$	This study
M_{milk}	$(10 - 0.02 \times d_m) \times f_{mL}$ $=555.6 - 1.1 \times d_m$	This study
M_p	$1.8 \times m_{milk} + 5.2 \times 10^{-7} \times d_g^3$	This study
M_{resp}	$f_{mcm} \times f_{mind} \times 0.0189 \times \exp(0.537 \times(2.966 + 0.00069 \times T_{av}^2 + 0.0218 \times T_{av}))$ $= 81.65 \times \exp(0.537 \times(2.966 + 0.00069 \times T_{av}^2 + 0.0218 \times T_{av}))$	Stevens[27]
M_{oral}	$2/3 \times M_{resp}$	Kohn[2]
M_{nasal}	$1/3 \times M_{resp}$	Kohn[2]
M_{urea}	$m_{dry} \times D \times E_{ex} \times C_p \times 0.06$	Kohn[2]
S	$0.09 \times m_{animal}^{0.67}$	McGovern et al.[40]

Table S4: Modelled contributions of each flux (%) to total amount of O input or output flux for the whole year, the grazing seasons and the stall seasons. The values are presented as mean ± SD.

	Contribution (%)	Whole year	Grazing seasons	Stall seasons
Input fluxes	Drinking water	60.3 ± 9.3	54.0 ± 7.9	68.1 ± 2.6
	Feed moisture	26.0 ± 10.0	33.3 ± 7.5	16.8 ± 1.6
	Air O uptake	8.5 ± 1.2	7.7 ± 0.5	9.5 ±1.0
	Chemically bound O	4.3 ± 0.7	3.9 ± 0.5	4.8 ± 0.5
	Air vapor	1.0 ± 0.3	1.1 ± 0.2	0.7 ± 0.2
Output fluxes	Fecal, urinary and sweat water	67.1 ± 6.8	64.3 ± 7.5	70.6 ± 3.4
	Orally exhaled water	8.2 ± 1.0	8.3 ± 0.8	8.2 ± 1.3
	CO_2 production	7.9 ± 1.1	7.1 ± 0.5	8.8 ± 1.0
	Transcutaneous vapor	6.4 ± 5.2	9.4 ± 4.9	2.6 ± 1.8
	Milk water	6.2 ± 4.9	6.6 ± 4.6	5.6 ± 5.1
	Nasally exhaled water	4.1± 0.5	4.1± 0.4	4.0 ± 0.6
	Organic products	0.3 ± 0.1	0.2 ±0.1	0.3 ± 0.1
	Urea	0.1 ± 0.0	0.1 ± 0.0	0.1± 0.0

Table S5: Overview of O input fluxes.

Input	Mean amount (mole)	Mean $\delta^{18}O$ (‰)	Range of $\delta^{18}O$ (‰)	Source of $\delta^{18}O$
Drinking water	2359	-10	-9.5 to -10.5	Measured
Leaf water intake	1015	0	-11 to 9	Measured and MuSICA modeled[41]
Silage water intake	594	-8	-12 to 14	Sun et al.[17]
Air O uptake	330	15.1	15.1 to 15.1	Kohn[2]
Chemically bound O	168	24	20 to 27	MuSICA modeled[41]
Stem water intake	113	-9	-13 to -7	Measured
Air vapor	39	-18	-26 to -12	Measured
Water adhering to leafs	32	-15	-15 to -2	From measured precipitation

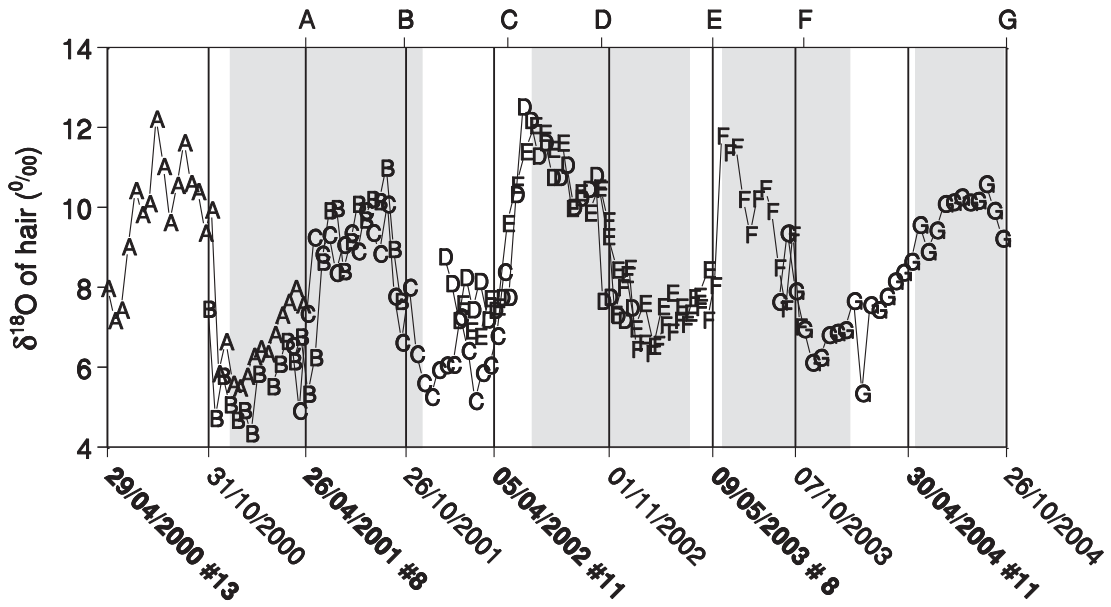

Fig. S1: The $\delta^{18}O$ of tail switch hairs and keeping conditions during five years. The upper x axis labels indicate the time of sampling tail hairs and the respective letters are used as markers for the $\delta^{18}O$ data of every 1-cm piece of hair from the root of the hair at the sampling date to the hair tip as detailed in Materials and Methods, section 'position-time assignment of hair segment data'. Vertical lines and lower x axis labels show times of grazing/keeping shifts (bold labels followed by the paddock number indicate start of grazing; normal labels indicate start of stall seasons). Grey shaded areas denote periods when the cow fed a calf.

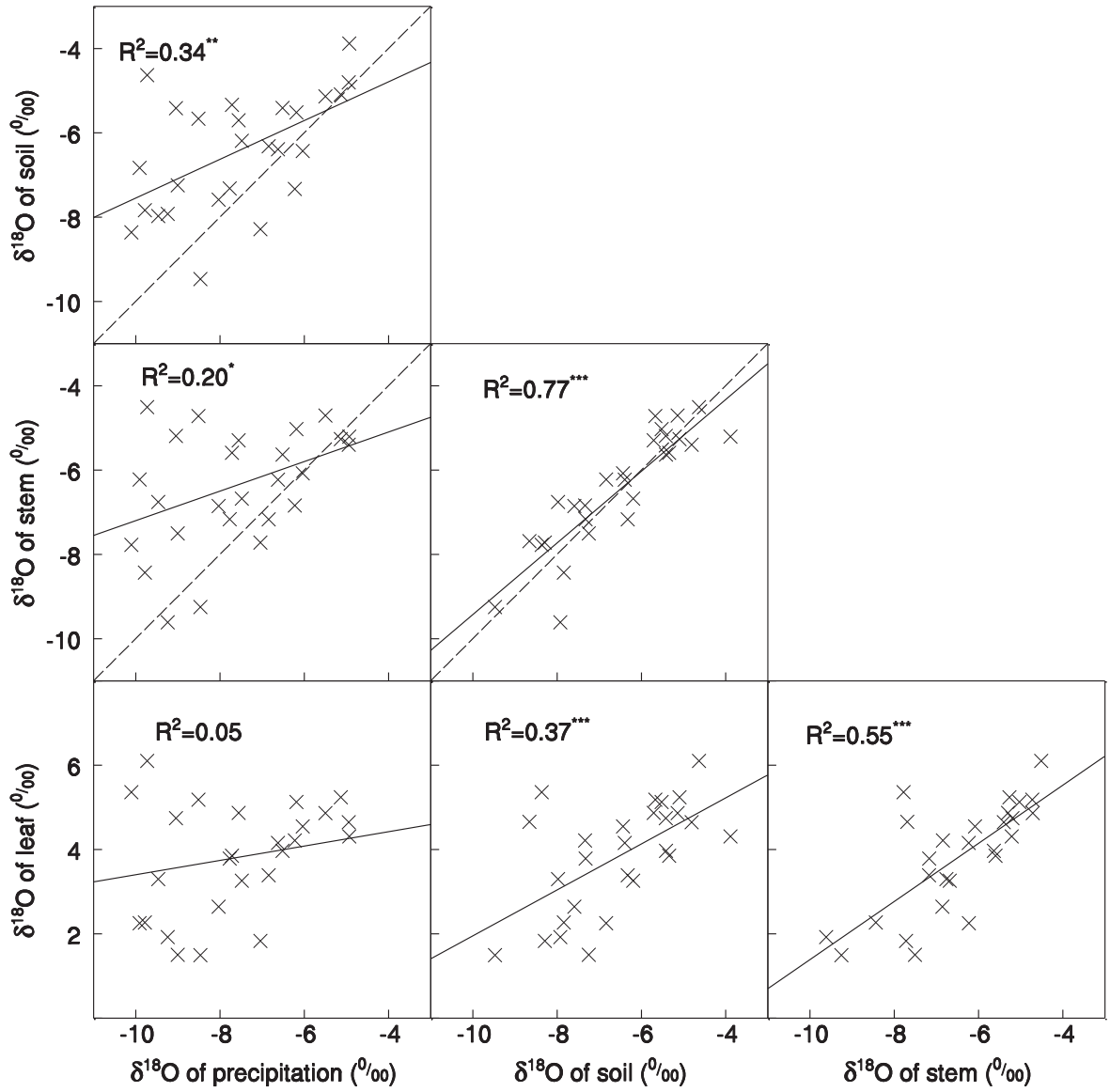

Fig. S2: Relation among average monthly $\delta^{18}O$ of soil water, stem water, leaf water and precipitation of measurements at midday from 2006 to 2012 (N=27 for each item). Solid and dashed lines represent linear regressions and 1:1 lines, respectively. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

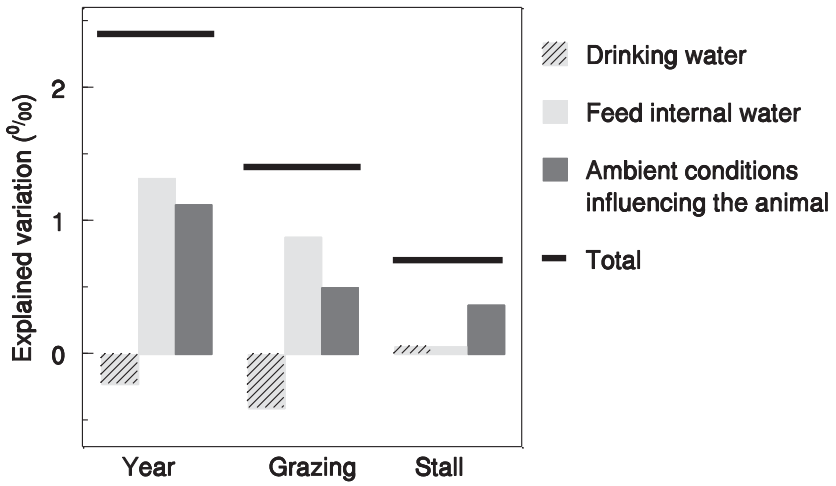

Fig. S3: Explained variation of δ_{hair} by different parameters in whole years, grazing and stall seasons (the parameters contributing little to the variation are not shown here). Lines represent the total variation of $\delta^{18}O$ in hair in whole years, grazing and stall seasons. Note that the effect of ambient conditions influencing the animal does not include the effects on plants. The sum of feed and ambient conditions is larger than total variation because drinking water intake compensates some of both effects.

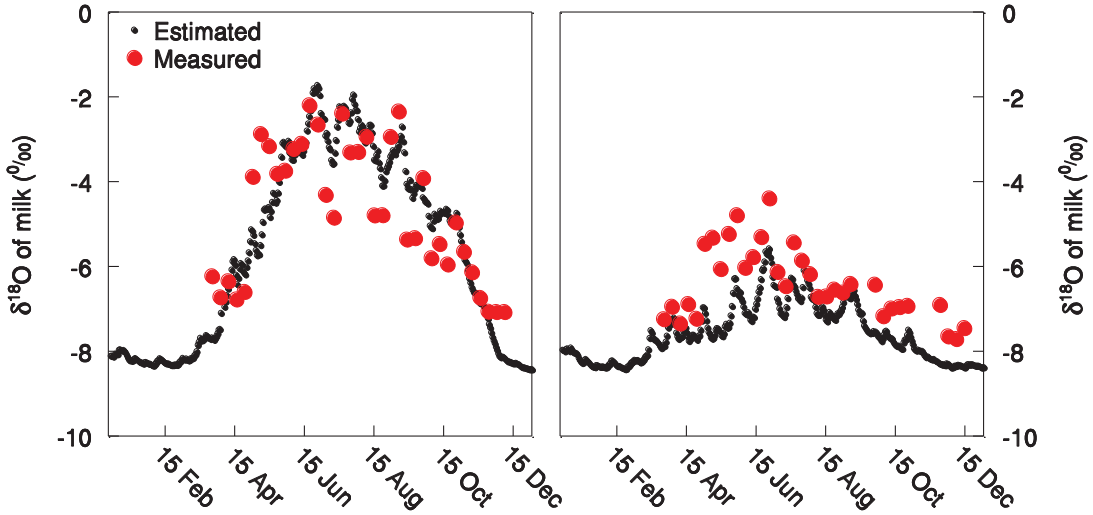

Fig. S4: Measured and predicted $\delta^{18}O$ in milk water during a year for two farms differing considerably in milk yield: Left farm: 14 kg d^{-1} annual average per cow; right farm: 26 kg d^{-1} annual average per cow. Note: the large difference between both farms is not caused by the difference in milk yield, which has a marginal influence, but it is caused by the exclusive provision of fresh grass in the left farm during the growing season while the right farm provides no fresh grass but constant feed (total mixed ration) throughout the year.

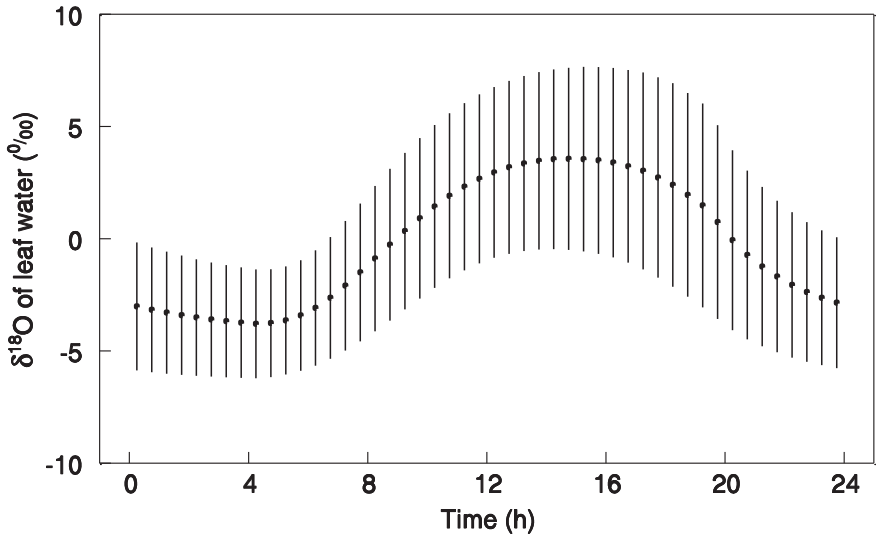

Fig. S5: Diurnal of $\delta^{18}O$ in leaf water estimated by MuSICA. Note: points and lines denote average values and standard deviations during five grazing seasons.

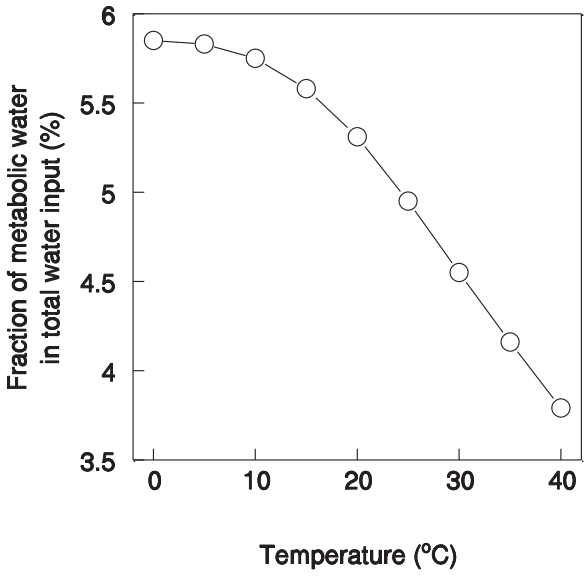

Fig. S6: Influence of temperature on the fraction of metabolic water as predicted by the MK model under otherwise constant conditions.