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Emergence and occurrence of performance-enhancing substance use in Australia determined by wastewater analysis

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Steroidal androgens, non-steroidal androgens and other non-steroidal performance- and image-enhancing drugs that are Schedule III substances under the US Controlled Substances Act as well as Schedule 4 and 10 substances under the Australian Poisons Standard are being used by athletes and non-athletes. Recent data suggest that they are growing in popularity among those communities. However, more information is necessary to understand the extent of their use by the general population. This study provides more information by assessing the emergence and occurrence (temporal and spatial trends) of performance- and image-enhancing drug use within the general community through wastewater analysis. For this, archived wastewater collected from 2009 to 2021 from one treatment plant and wastewater collected in August 2021 from 51 treatment plants covering 11.6 million Australian people (45% of the national population) was extracted and analysed for 52 performance- and image-enhancing drugs. These included steroidal and non-steroidal androgens, peroxisome proliferator-activated receptor delta agonist metabolites, ReV-ErbA agonists, β_2-agonists, myostatin inhibitors and growth hormone secretagogues. Analysis of the samples from 2009 to 2021 showed the earliest detection of a non-steroidal androgen, enobosarm, in 2011, followed by cardarine metabolites, ibutamoren and ligandrol and testolone in 2014, 2016 and 2017, respectively. The concentrations of all identified substances increased until 2021 following their first detection. Steroidal androgens and non-steroidal performance-enhancing drugs were detected in samples from 49 of the 51 investigated wastewater treatment plants. A higher number of different analytes were detected in samples representing catchments with larger populations. Our study demonstrates that wastewater analysis can be a useful tool for providing information on performance-enhancing drug use in the general population.

Steroidal and non-steroidal performance- and image-enhancing drug (PIED) use to improve performance and physical appearance extends beyond athletes to the general population. They are being used despite their known long-term, negative health side-effects, consequently raising concerns for public health[1–3]. Despite being Schedule 4 and 10 substances under the Australian Poisons Standard, not being approved by the US Food and Drug Administration (FDA), not yet having cleared any clinical trials[4] and being prohibited by the World Anti-Doping Code[5], non-steroidal androgens and PIEDs, for example, selective androgen receptor modulators (SARMs), have recently gained popularity among the athletic community[4,6–8], suggesting that their widespread use within the general population may consequently follow. An early indicator is

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the significant increase in Google searches for the keyword 'SARMs' between 2015 and 2021[9]. Studies have shown that many products bought online claiming to contain certain PIEDs often contained incorrect ones, a mixture of multiple PIEDs or different concentrations to those stated on the label, potentially posing a health risk to users[10–13]. The attention of researchers and policymakers is warranted, yet the extent of PIED use, especially among the general public, is unknown[10].

Wastewater analysis has recently become popular and widely used for monitoring SARS-CoV-2 in the general population[14–17], but before this, it was used extensively to monitor licit and illicit drug use worldwide[18–21]. It has also recently been used to study the influences of sociodemographics on chemical consumption[22]. In this study, we used retrospective wastewater analysis to investigate the emergence, occurrence and trends of PIED use in the Australian population. Archived wastewater collected from 2009 to 2021 from one treatment plant and wastewater collected in August 2021 from 51 treatment plants covering 11.6 million Australian people (45% of the national population) was extracted and analysed for 52 PIEDs using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Performance- and image-enhancing drugs analyzed in this study included steroidal and non-steroidal androgens, peroxisome proliferator-activated receptor delta agonist metabolites, ReV-ErbA agonists, β_2-agonists, myostatin inhibitors and growth hormone secretagogues.

Emergence of non-steroidal PIEDs from 2009 to 2021

In total, 16 of the 52 investigated steroidal and non-steroidal PIEDs were detected. The steroidal androgens, hormones and metabolites 19-norandrosterone, androstenedione, androsterone, boldenone, 17β-hydroxy-5β-androst-1-en-3-one (boldenone metabolite), epitestosterone, metandienone, progesterone, testosterone and stanozolol were detected in the archived samples. Apart from boldenone, androstenedione and testosterone, they did not appear to show any obvious trends over the 13-year period, with their concentrations in wastewater fluctuating within a certain range (see Supplementary Fig. 1 and Supplementary Table 5 for details). Boldenone, androstenedione and testosterone showed an upwards trend from 2018 to 2020.

Detected non-steroidal PIEDs were cardarine (also known as GW501516, Endurobol and GSK-516) metabolites cardarine sulfone and cardarine sulfoxide, enobosarm (also known as ostarine, MK-2866, S-22 and GTx-024), ibutamoren (also known as MK-677), ligandrol (also known as LGD-4033) and testolone (also known as RAD140; Fig. 1). Enobosarm was the first non-steroidal PIED to appear in the temporal samples, emerging as early as 2011. After this, the concentration increased steadily until 2021, with samples from 2018 and 2019 having higher concentrations than expected if the increase had remained linear. Both cardarine metabolites were first detected at very low concentrations in samples from 2014 and 2016, after which their concentrations steadily and almost linearly increased until 2021. A peak was seen in 2018 that was about four times the concentration of the previous and following year. Ligandrol was only detected from 2017 onwards, but followed the same pattern as enobosarm. Ibutamoren was first detected in 2016, after which its concentration increased sharply. Although the concentration decreased again in 2019 and 2020, it increased in 2021 to the same level as during the peak in 2018. Testolone, first detected in 2017, is the only non-steroidal PIED that had the highest concentration in 2021 instead of 2018. Apart from 2018 and 2019, where concentrations were particularly high compared with other years, generally, all non-steroidal PIED concentrations increased after their first detection.

Spatial trends of PIEDs in the general community

The endogenous steroids and hormones androstenedione, androsterone, epitestosterone, progesterone and testosterone served as controls and were detected in all 51 samples. One exception was progesterone, which was below the limit of detection in one sample. Boldenone and its metabolite were detected at 45 and 46 of the 51 sites, respectively (Supplementary Table 6a–c). A total of nine synthetic PIEDs and metabolites were detected across all samples: the steroidal androgens metandienone, oxandrolone and stanozolol, and the non-steroidal PIEDs cardarine sulfone, cardarine sulfoxide, enobosarm, ibutamoren, ligandrol and testolone. Overall, the non-steroidal PIEDs were detected more frequently than the steroidal androgens, with ibutamoren (at 46 of the 51 sites (hereafter denoted as 46/51)), ligandrol (44/51), testolone (44/51) and enobosarm (40/51) having the highest detection rates (Fig. 2a).

The per capita mass loads of the synthetic PIEDs in Fig. 2a are arranged by the size of the population served by the wastewater treatment plant (WWTP). A difference in the number of different PIEDs detected was observed, depending on the size of the population. In populations of >150,000 ($N = 17$), an average of 6.9 out of the 8 different synthetic PIEDs were detected. With decreasing population, the average number of detections decreased: 6 out of 8 for populations of 30,000 to 150,000 ($N = 17$) and 3.4 out of 8 for populations below 30,000 ($N = 17$). Samples from two catchments, both covering a population of less than 30,000, showed no detection of synthetic PIEDs. As the two cardarine metabolites originate from the same parent compound (cardarine), they were only counted as one 'different PIED' when both were detected in the same sample.

Stanozolol, enobosarm and ibutamoren had the highest per capita mass loads and the largest differences between catchments (Fig. 2b). On average, with less than 0.25 mg per day per 1,000 people, metandienone, cardarine sulfone, cardarine sulfoxide and testolone had the lowest per capita mass loads (Fig. 2c). Several steroidal androgens and metabolites (Supplementary Information), non-steroidal androgens andarine (also known as S-4 and GTx-007) and S-23, ReV-ErbA agonist SR9009 (also known as stenabolic) and its metabolites M2 and M6 (ref. 23), β_2-agonist clenbuterol and myostatin inhibitor YK-11 were not detected in any of the samples.

Temporal trends of non-steroidal PIEDs

This investigation shows the application of wastewater analysis to report non-steroidal PIED use among the general community, demonstrating its suitability as a complementary source of exposure and use data. The suitability and applicability of wastewater analysis is supported by the closeness of the concentrations in the two independent pools, demonstrating the reproducibility of this approach. Retrospective analysis of samples collected from 2009 to 2021 revealed the emergence of non-steroidal androgens, peroxisome proliferator-activated receptor delta (PPAR-δ) agonist metabolites and the growth hormone secretagogue ibutamoren. While the earliest non-steroidal PIED to be detected was enobosarm in 2011, most of the others were first detected in 2014, 2016 and 2017, demonstrating that the use of many non-steroidal PIEDs by the general population has only increased in recent years to a degree that allows surveillance of trends using wastewater analysis. The increasing trend from first detection to 2021 supports claims of growing interest in these non-FDA-approved and therefore clandestinely produced and sold substances among PIED users. This reinforces the concern that the consumption of these substances is an emerging public health issue.

The emergence of multiple non-steroidal PIEDs around the same time may be a result of polysubstance use, an increasing number of users or doses, a shift from the historically more commonly used steroidal androgens or a combination of any of these, but cannot be determined using wastewater data. Nevertheless, this study demonstrates that wastewater analysis can be a useful complementary source of data for understanding PIED use in the general population in addition to surveys, anti-doping and seizure data.

No trends were observed for the detected steroidal androgens aside from boldenone, androstenedione and testosterone, which showed an increase from 2018 to 2020. Backe et al.[24] reported in 2011 that they observed similar temporal concentration profiles for

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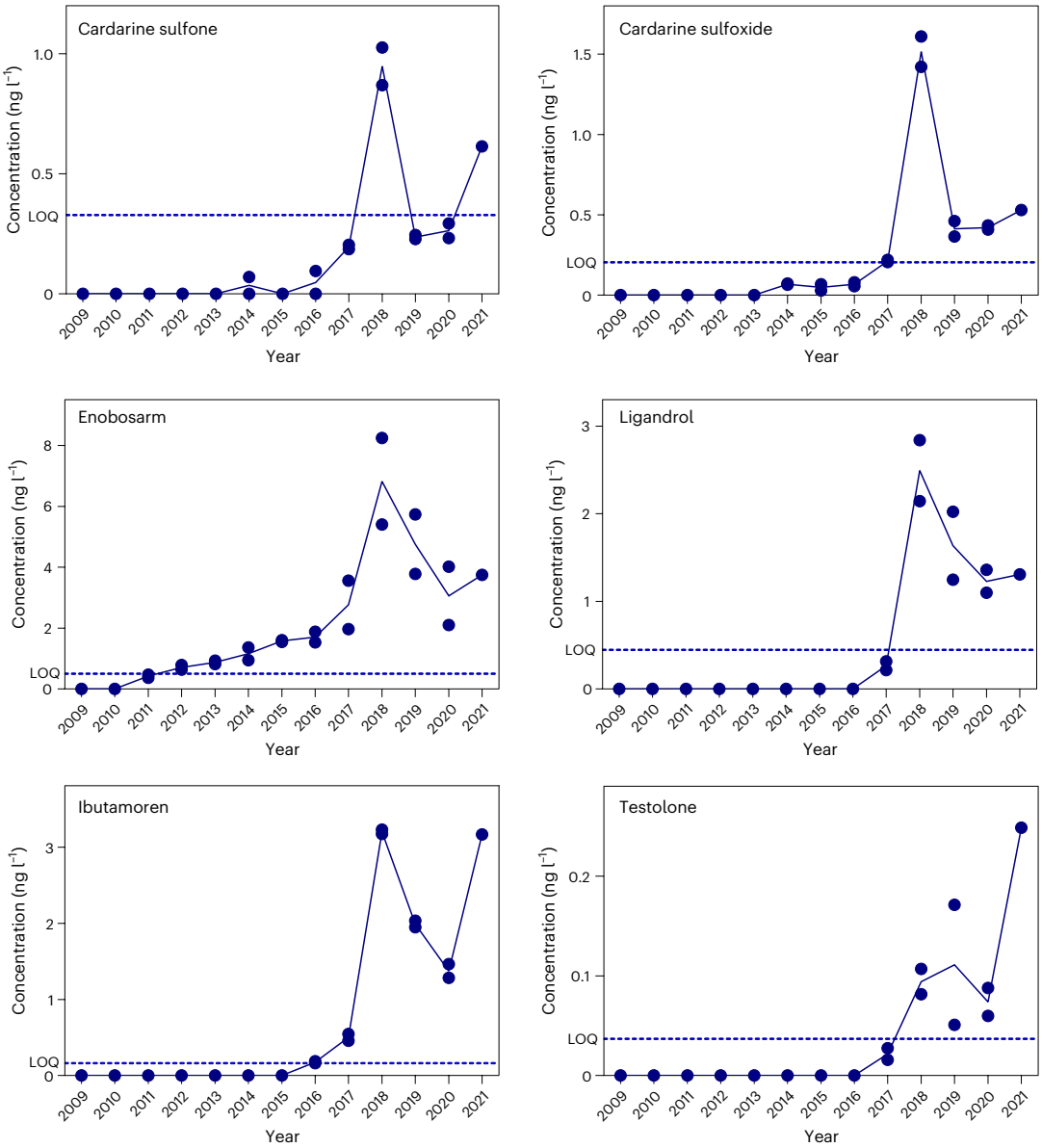

Fig. 1 | Emergence of non-steroidal PIEDs from 2009 to 2021. Concentrations of cardarine sulfone, cardarine sulfoxide, enobosarm, ligandrol, ibutamoren and testolone in archived wastewater samples collected from one wastewater treatment plant from 2009 to 2021. Each point represents a pooled sample for that year (pool of one sample per month ($N = 12$), two separate pools per year). The blue dashed lines represent the method limit of quantification (LOQ) for each analyte. The solid lines run through the mean of the two pools. Note, the data for 2009 comprises samples from only November and December ($N = 4$); the data for 2021 were taken from spatial analysis (one pooled week in August 2021, $N = 7$). The years 2010–2020 are pooled samples of $N = 12$.

boldenone, androstenedione and testosterone in wastewater influent samples, suggesting a connection between boldenone and testosterone loads. It was not determined whether the boldenone originated from a different compound converted in situ, from illicit use or from an unknown source. As in this study a similar increase in boldenone's metabolite was not observed, it is unlikely that this increase was caused by a higher consumption of boldenone during these 3 years.

It can be assumed that the in-sewer stability of PIEDs is less likely to have had an influence on the trends due to the samples originating from the same catchment with the same or similar hydraulic retention time. In-sample stability, however, should be considered in the interpretation of the data. A previous study showed that the ten detected steroidal androgens, hormones and metabolites were all stable in wastewater past 200 days when acidified with hydrochloric acid and stored at −20 °C (ref. 25; the same sample preparation as in this study). While this does not exclude the possibility of degradation past 200 days, it does indicate that these chemicals have sufficient stability for retrospective analysis under those conditions. All detected non-steroidal PIEDs were also stable past 200 days[25] (ibutamoren was not assessed in the study). One exception was cardarine sulfoxide (50% transformation after 164 days). It is therefore possible that cardarine may have been used before its first detection in 2014 and that the overall level of use was higher than observed.

In the World Anti-Doping Agency's (WADA's) Anti-Doping Testing Figures Reports from 2009 to 2021[26], cardarine was first reported in 2015, although it is possible that its first reporting as an Adverse Analytical Finding (AAF) was in 2012. This is because WADA did not specify cardarine from 2012 to 2014, but rather reported AAFs of PPAR-δ agonists. The first reported AAF of a SARM was in 2010 (andarine), while ibutamoren was first reported in 2015 and testolone in 2016. This is consistent with the order of detection of the drug classes in this study, where the first was a SARM (enobosarm) in 2011, followed

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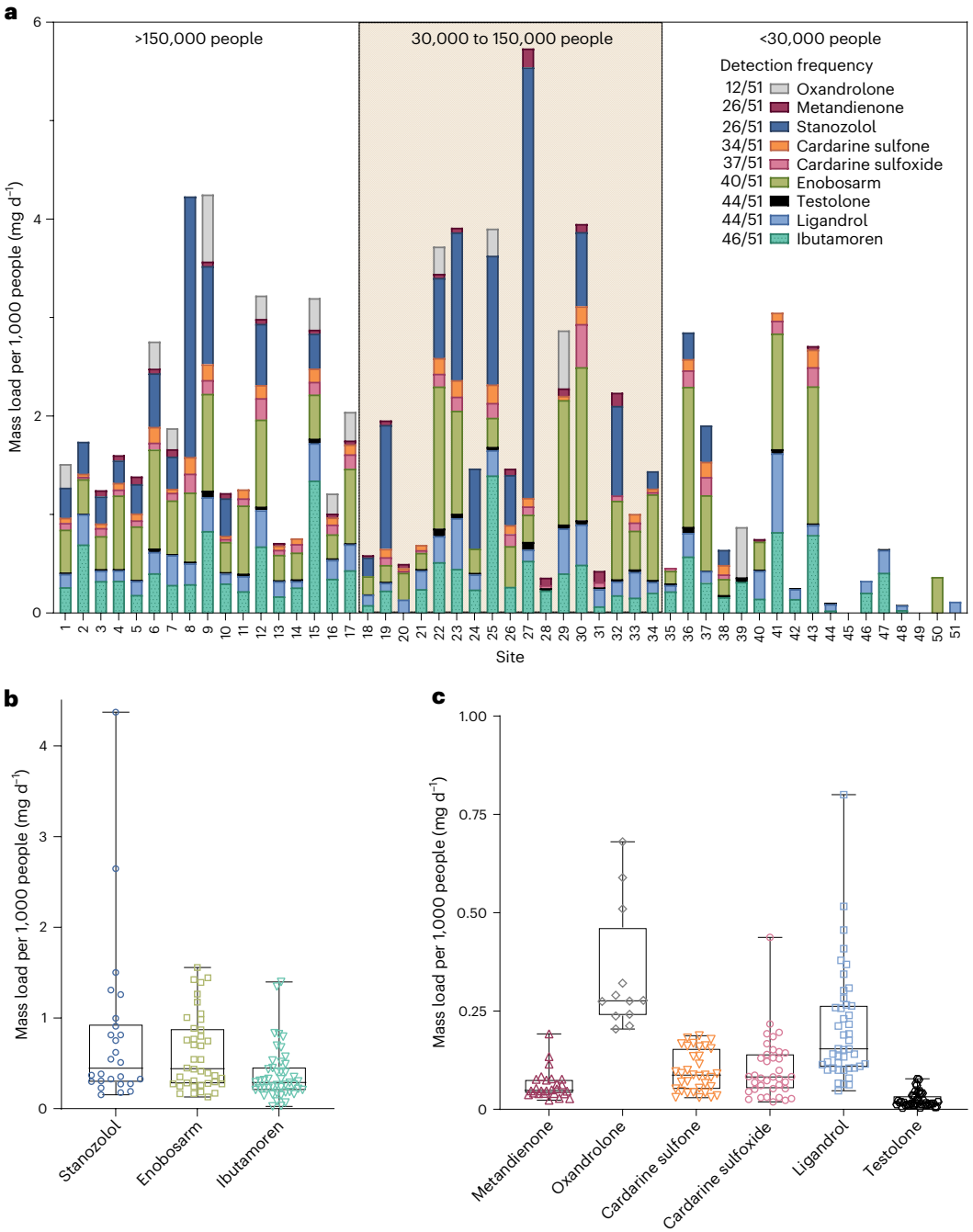

Fig. 2 | Occurrence of PIEDs across Australia. Per capita mass load data from wastewater analysis of samples collected from 51 sites across all Australian states and territories except Western Australia. **a**, The mass load data for the 51 sites are sorted by decreasing population size along the *x* axis. The detection frequencies are listed in the figure legend. **b**,**c**, Box plots of PIEDs with the highest (**b**) and lowest (**c**) per capita mass loads. The box plots show all points (n = detection frequency for each analyte at the 51 sites, shown in **a**). The whiskers show the minima and maxima, the boxes extend from the 25th to 75th percentiles and the centre lines represent the median. The box plots were generated using GraphPad Prism (v.9.3.1).

by cardarine metabolites in 2014, ibutamoren in 2016 and testolone in 2017 (Fig. 3). An increasing trend for the non-steroidal PIED AAFs can also be seen after the first report of their detection. WADA's reported AAFs (including only the substances investigated in this study) from 2009 to 2021 are summarized in Supplementary Table 7. The data reveal similarities in the first detection and trends of PIEDs in the Australian general population (>150,000 people) investigated in this study and among international athletes tested by WADA accredited laboratories (Fig. 3).

Spatial trends of PIEDs

Multiple types of PIEDs were detected across 96% (49/51) of the investigated 51 WWTPs covering nearly half (45%) of the Australian population in August 2021. This supports claims of the popularity of non-steroidal PIEDs among the general community[9,13,27]. The widespread detection of substances that are 'not for human consumption'[4,13] is a cause for public health concern. In particular, non-steroidal androgens, PPAR-δ agonist metabolites and the growth hormone secretagogue ibutamoren were the most frequently detected, which is consistent with the most

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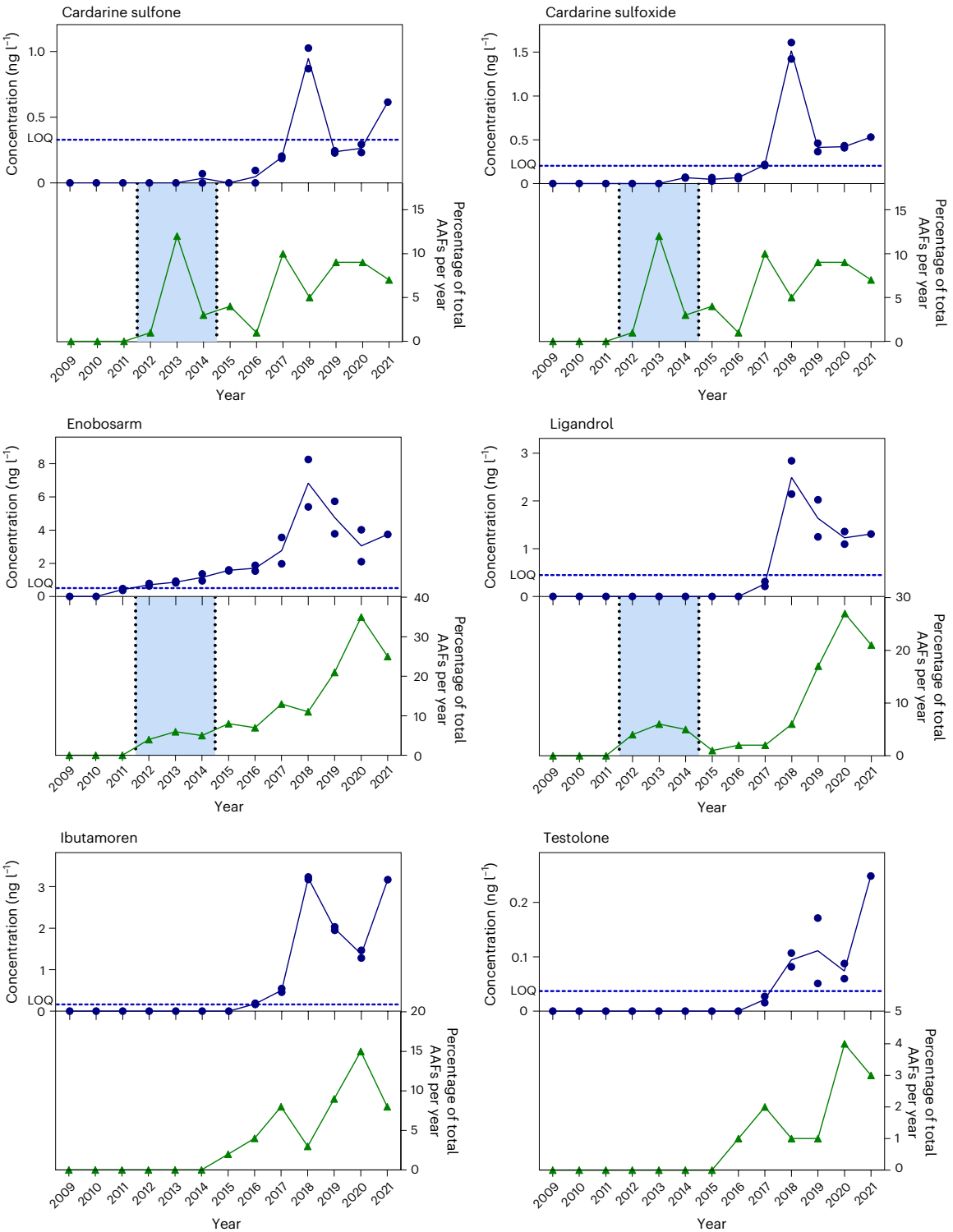

Fig. 3 | Comparison of non-steroidal PIEDs in wastewater and AAFs for the same PIEDs reported by WADA from 2009 to 2021. Concentrations of the non-steroidal PIEDs cardarine sulfone, cardarine sulfoxide, enobosarm, ligandrol, ibutamoren and testolone quantified in wastewater in this study (top) compared with the AAFs for the same non-steroidal PIEDs as a percentage of total AAFs in each drug class reported in WADA's Anti-Doping Testing Figures Reports[26] (bottom) from 2009 to 2021. The blue shading in the graphs for cardarine sulfone and cardarine sulfoxide indicates years in which WADA reported PPAR-δ agonist, not cardarine; the blue shading in the graphs for enobosarm and ligandrol indicates years in which WADA reported SARMs, not enobosarm or ligandrol. The horizontal dashed lines represent the limit of quantification (LOQ).

common substances found in US (2016) and Australian (2017–2018) studies that investigated products bought online claiming to contain non-steroidal PIEDs[10,11]. In addition, the three most used non-steroidal androgens according to an internet-based survey in 2020[28] (ligandrol (56%), enobosarm (54%) and testolone (41%)) are consistent with the most frequently detected non-steroidal androgens in this study.

The greater variety of PIEDs in larger populations can likely be attributed to a higher number of users and therefore a greater

variability in access to certain types, cycle stage (on/off use), purpose/desired effect of PIED use, and so on. Furthermore, contaminated online-bought products[10,11,13] and common polysubstance use and 'stacking' among users[29–31] are likely contributing factors to the widespread detection of multiple PIEDs across 51 populations.

This study has several strengths. The developed and validated analytical method is extremely sensitive for non-steroidal PIEDs (limits of detection: 0.3 ± 0.37 ng l^{-1} non-steroidal PIEDs (n = 13) and 12.8 ± 63.8 ng l^{-1} steroidal PIEDs (n = 36)), enabling the detection of trace amounts in wastewater. The unique archive of temporal samples allowed retrospective analysis of PIED use going back 13 years, and the results of the independent pools showed method reproducibility. In addition, the spatial samples from 51 WWTPs cover 11.6 million people, almost half of the Australian population.

Limitations

Overall, there was a lack of detection of steroidal androgens, in particular, of their metabolites. The lower detection frequency of steroidal androgens, however, does not necessarily equate to lower popularity or use. Steroidal androgens are extensively metabolized, so higher levels of metabolites compared with the parent compounds are expected in urine, and hence in wastewater. While relevant metabolites were included in this study, the sensitivity of the analytical method for these metabolites is generally lower than for the parent analytes and their concentrations may simply not have been high enough to overcome this challenge.

Due to analyte stability (in the sewer and after collection) and a lack of excretion data, calculated mass loads in this study may underestimate consumed and excreted amounts of PIEDs and should be seen as conservative estimates[25,32,33]. The detection of PIEDs is linked to their stability and the detection limits of the analytical methods, and non-detection does not rule out the use of those substances in the population. For example, ReV-ErbA agonist SR9009 and its metabolites are unstable in the sewer[32], and if present at low concentrations likely degrade to levels below the detection limit before the wastewater is collected.

Importantly, the temporal study and its results are only a reflection of PIED use within one WWTP catchment in Australia. Trends and emergence patterns may differ for other areas, which requires further investigation. In this study, emergence describes the first detection of non-steroidal PIEDs above detection limits and therefore potential previous use cannot be ruled out.

Conclusions

Retrospective analysis of archived wastewater samples revealed the emergence of the non-steroidal androgen enobosarm in 2011 with other non-steroidal PIEDs subsequently emerging in 2014, 2016 and 2017. The increase in the concentrations of these substances after their first detection supports claims of increasing use among the public. Occurrence data revealed widespread use of these substances in the general community with the presence of at least one performance-enhancing substance in 49 of the 51 studied populations.

Methods

Temporal and spatial samples

For both parts of this study, composite 24-h wastewater influent samples were collected by WWTP personnel, preserved with hydrochloric acid (HCl) to pH 2 and stored at −20 °C until analysis. For the temporal (emergence) study, samples were collected between 2009 and 2021 from a single WWTP that serves a population of >150,000 people. One sample per month was randomly selected from weekly archived samples and pooled by year (that is, 12 samples per year were combined to form 1 sample per year). The samples were selected, defrosted and pooled with equal volume. A second pooled sample for the same year was prepared in the same way from separate weekly samples, resulting in an independent second pool. Every day of the week was represented at least once in each pool. Exceptions were 2009, where only samples from November and December were available, and 2021, where spatial data for that treatment plant catchment were used.

For the spatial (occurrence) study, 24-h composite influent wastewater samples were collected from 51 WWTPs during the Australian Census week in August 2021. These 51 WWTPs cover approximately 11.6 million people (45% of the Australian population) from all states and territories except Western Australia. Daily composite samples were collected using site-specific optimized autosamplers for 4–7 days at each site and immediately preserved with HCl to pH 2 and frozen (−20 °C, storage for 4 months until analysis). The flow data (in megalitres) during that period were provided by each WWTP. The wastewater influent samples were defrosted, homogenized and samples from all days per site were pooled in equal volumes to a total of 10 ml.

Sample preparation and analytical method

The PIEDs investigated in this study included steroidal androgens, non-steroidal androgens (that is, SARMs) and other non-steroidal PIEDs (that is, PPAR-δ agonists, growth hormone secretagogues, ReV-ErbA agonists, β_2-agonists and myostatin inhibitors). A solid-phase extraction LC-ESI-MS/MS method was developed and validated for 52 PIEDs and metabolites using the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use[34] (a list of all analytes is provided in Supplementary Table 1 and validation results in Supplementary Tables 3 and 4). For sample preparation, 10 ml of HCl-preserved and unfiltered wastewater was fortified with 8 µl of an isotopically labelled standard mix (concentration of mix 25 µg l^{-1}, vortexed, centrifuged for 20 min at 5,251 g and extracted using 3-cm^3 Oasis MCX Waters) cartridges. The cartridges were conditioned with 3 ml methanol (MeOH) and 3 ml Milli-Q water at pH 2 (acidified with HCl). Then, 10 ml samples were loaded onto the cartridges under gravity conditions, which were then washed with 3 ml of 40% MeOH at pH 2 and 3 ml of 20% acetonitrile (ACN) at pH 2. After drying the cartridges under N_2 (using a positive pressure manifold for 1.5 h), they were eluted in three separate steps and each eluate was collected in separate 15 ml polypropylene centrifuge tubes (Sarstedt Australia). The first elution (eluate 1) was conducted with 3 ml ACN, the second elution (eluate 2) with 3 ml ACN + 5% ammonium hydroxide (NH_4OH) (28–30%) and the third elution (eluate 3) with 3 ml MeOH + 5% NH_4OH (28–30%).

The 3 ml eluates were then blown to dryness under N_2 at 35 °C in a water bath, after which 40 µl MeOH was added to the dried extracts. The tubes were then vortexed and 160 µl Milli-Q water at pH 2 was added (total volume 200 µl, concentration factor 50). The tubes were vortexed and centrifuged for 30 min at 5,251 g, after which the supernatant was transferred into 250 µl glass vial inserts. Milli-Q water blanks, duplicates and spiked samples were prepared every ten samples for quality control purposes. Spiked samples were fortified with 20 ng l^{-1} native PIEDs. The extracts were frozen at −20 °C until they were subjected to targeted analysis using LC-ESI-MS/MS.

To allow for comparison between different catchments in the spatial study, PIED concentrations were converted into mass loads in mg per day per 1,000 people in each WWTP catchment by multiplying the analyte concentration by the flow volume and dividing by the population[35,36] in 1,000s. For the pooled spatial samples, the average flow volume of samples contained in the pool was used. For the purposes of further data analysis and to de-identify the sites, catchments were categorized into three size categories based on population size[35,36] (updated for the population in 2021): >150,000, 30,000–150,000 and <30,000. Concentrations in the temporal study were not converted into mass loads as samples from the same WWTP were compared.

LC-ESI-MS/MS analysis. A Shimadzu Nexera LC-40 instrument was used for LC. A Kinetex 1.7 µm C18 100 Å 100 mm × 2.1 mm column with a SecurityGuard ULTRA C18 2.1 mm guard column (Phenomenex) and

a stainless-steel Restek UltraShield ultra-high-performance liquid chromatography pre-column filter (Fisher Scientific) was used for chromatographic separation. A pre-injection column (Kinetex 5 µm EVO C18 100 Å 30 mm × 2.1 mm, Phenomenex) was installed between the pump mixer and the injector. The oven temperature was set to 45 °C, the autosampler was set to 8 °C and the injection volume was 8 µl. Mobile phase A was 0.5 mM ammonium fluoride (NH_4F) in 95:5 Milli-Q water–methanol (v/v) and mobile phase B was 0.5 mM NH_4F in 95:5 methanol–Milli-Q water (v/v) at a flow rate of 0.4 ml min^{-1}. The LC program was as follows: 0–0.5 min 20% B, 0.5–1.5 min linear increase to 45% B, 1.5–15 min linear increase to 75% B, 15–16 min linear increase to 100% B, 16–19.9 min hold at 100% B, 19.9–20 min linear decrease to 20% B and finally 20–24 min hold at 20% B. The analytes were analyzed using a triple quadrupole SCIEX 7500 system mass spectrometer operating in both positive and negative ionization modes in the same run (polarity switching). Ionization was achieved by ESI. The optimized conditions were ion source gas 1 at 3.45 bar, ion source gas 2 at 5.17 bar, curtain gas at 2.76 bar, a source temperature of 475 °C, and a spray voltage of 2,700 V for positive ionization and 2,500 V for negative ionization. Ion source gas was compressed air and curtain gas was nitrogen. Simple *Q0* dissociation (vendor-specific setting) was activated and scheduled multiple reaction monitoring (sMRM) was applied (for sMRM conditions, see Supplementary Table 2).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data obtained during this study are provided in the Supplementary Information. To ensure anonymity of the WWTPs, population and flow data are not made available, but instead, mass loads are provided on a per capita basis or as raw concentrations.

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Author contributions

K.M.S., J.W.O'B., B.J.T., N.S., J.F.M. and K.V.T. designed the research; K.M.S. and J.W.O'B. performed the research; K.M.S., B.J.T. and R.S. analysed the data; K.M.S. and J.W.O'B. drafted the manuscript; K.M.S., J.W.O'B., B.J.T., L.B., C.G., R.S., N.S., J.F.M. and K.V.T. contributed to the interpretation of data, provided critical revisions to the draft manuscript and approved the final draft.

Competing interests

The authors declare no competing interest.

Additional information

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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Software and code

Policy information about availability of computer code

Data collection	SCIEX OS (version 3.1.0.16485) was used to collect experimental data. Population and flow data were organized in Excel.
Data analysis	SCIEX OS (version 3.1.0.16485) was used to calculate concentrations of analytes in samples based on peak areas. Excel was used for method validation, concentration, and per capita mass load calculations. GraphPad Prism 9 was used for data visualization.

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Data obtained during this study are provided in the Supplementary Information. To ensure anonymity of the wastewater treatment plants, population and flow data are not made available, but instead, mass loads are provided on a per capita basis or as raw concentrations.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	Sex/gender-based data were not considered in this study.
Population characteristics	N.A.
Recruitment	N.A.
Ethics oversight	N.A.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Study description	The aim of this study was to assess emergence and occurrence of performance- and image-enhancing drug use within the general community through wastewater analysis. For this, archived wastewater collected from 2009 to 2021 from one catchment serving >150,000 people and wastewater from 51 treatment plants covering 11.6 million Australian people (45% of the national population) was extracted. The extracts were analyzed for 52 performance- and image-enhancing drugs including steroidal and nonsteroidal androgens, peroxisome proliferator-activated receptor delta agonist metabolites, ReV-ErbA agonists, beta-agonists, myostatin inhibitors, and growth hormone secretagogues. This study showed the potential of wastewater analysis for performance- and image-enhancing drug use monitoring and revealed the types and amounts used in Australia.
Research sample	The samples used in this study were pooled 24-hr composite wastewater influent samples from wastewater treatment plants (N=51). This type of sample contains the pooled excretions of entire communities served by the wastewater treatment plants.
Sampling strategy	For the temporal study, 24-hr composite samples were collected between 2009 and 2021 from a single wastewater treatment plant that serves a population of >150,000 people. One sample per month was randomly selected from weekly archived samples, and pooled by year (e.g, 12 samples per year combined into one per year). Samples were defrosted and pooled by equal volume. A second pooled sample for the same year was prepared in the same way from separate/different weekly samples, resulting in an independent second pool (N=12 per year). Every day of the week was represented at least once in each pool. Exceptions were 2009, where only November and December samples were available, and 2021, where spatial data for that treatment plant catchment were used. For the spatial study, 24-hr composite influent wastewater samples were collected from 51 wastewater treatment plants from all states and territories except Western Australia. Daily composite samples were collected using site specific optimized autosamplers for four to seven days at each site. The wastewater influent samples were defrosted and all days per site were pooled in equal volumes to a total volume of 10 mL.
Data collection	Experimental data was collected using SCIEX OS (version 3.1.0.16485). Flow data was provided by the wastewater treatment plant operators. Population data were collected by the Australian Bureau of Statistics and refined using catchment maps (Tscharke, B. J. et al. Harnessing the Power of the Census: Characterizing Wastewater Treatment Plant Catchment Populations for Wastewater-Based Epidemiology. Environ. Sci. Technol. 53, 10303-10311, doi:10.1021/acs.est.9b03447 (2019).).
Timing and spatial scale	Wastewater samples used for the temporal study were collected on a weekly basis from November 2009 to August 2021. Samples for the spatial study were collected during the Australian Census week in August 2021 and represented a total population of approximately 11.6 million people (45% of the Australian population). These samples were collected under the Australian National Wastewater Drug Monitoring Programme (NWDMP).
Data exclusions	No data were excluded from analyses.
Reproducibility	Both instrument and extraction method were validated following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. Precision and accuracy data were determined using 8 repeat injections at 5 different concentrations for the instrument method and 7 injections of separate extracts at 3 different concentrations for the extraction method. The same wastewater used for method validation was extracted during each sample extraction batch to ensure reproducibility.

Randomization	For each sample, eluate 1 was injected followed by eluate 2 before the next sample was injected. All eluate 3 were run together in a separate batch. The temporal samples from 2009 to 2020 were injected in a randomized order to avoid any potential instrument bias in the trend caused during the analytical run.
Blinding	The sample names consisted of site codes randomized by the Queensland Alliance for Environmental Health Sciences and were entered into the batch in that format for data acquisition. Site codes were matched to population and flow data after the concentrations were obtained.

Did the study involve field work? ☐ Yes ☒ No

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☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
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